

**UC Davis**

**UC Davis Electronic Theses and Dissertations**

**Title**

Prospects for Improving Alfalfa Yield Using Genomic- and Phenomic-Based Breeding

**Permalink**

<https://escholarship.org/uc/item/09h7c302>

**Author**

Francis, Matt William

**Publication Date**

2024

Peer reviewed|Thesis/dissertation

Prospects for Improving Alfalfa Yield Using Genomic- and Phenomic-Based Breeding

By

MATT FRANCIS  
DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Horticulture and Agronomy

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

Approved:

---

E. Charles Brummer, Chair

---

Steven J. Knapp

---

Allen Van Deynze

Committee in Charge

2024

# Abstract

The primary goal of the research presented in this dissertation is to assess the prospects of utilizing modern plant breeding methodology to address the lack of biomass yield improvement in alfalfa. Biomass yield improvement in alfalfa has remained stagnant for the last ~30 years and cultivated areas are in steady decline. Significant advancements have been made in genomics and phenomics in recent years with applications in plant breeding. Chapter 1 reviews relevant literature including the role alfalfa plays in western agriculture, history, biology, breeding, and an introduction to genomic selection and high throughput phenotyping. Chapter 2 seeks to empirically test genomic selection for forage dry matter yield in alfalfa. Genomic selection has proven successful for increasing the rate of genetic gain in other crops, and models have been developed for a variety of traits in alfalfa. However, no practical testing has occurred to date. Chapter 3 builds on the findings from the previous chapter by addressing some limitations in the predictive model. Namely, building a model based on family bulks and half-sib family performance, rather than on an individual plant basis, to better reflect the commercial environment alfalfa is grown in. The results from chapter 2 and 3 provide encouraging prospects with prediction accuracies around 0.3. However, one of the greatest limitations for a successful genomic selection breeding program is the size of the training population, particularly in crops with limited resources such as alfalfa. Chapter 4 explores the potential to greatly increase the size of breeding trials without a comparable increase in costs. We utilized drone based multispectral imagery to estimate biomass of alfalfa and a range of forage grasses across a range of plot types typically used in forage breeding trials. Remote sensing and high throughput phenotyping massively reduce the labor component of data collection while simultaneously providing accurate estimates of forage biomass.

# Contents

Chapter 1: Literature Review.....	1
Chapter 2: An Empirical Test of Genomic Selection for Forage Dry Matter Yield in Alfalfa.....	19
Chapter 3: Genomic Selection for Forage Dry Matter Yield in Alfalfa Using Family Bults.....	40
Chapter 4: Remote Sensing for Biomass Yield in Forage Breeding Plots.....	66

# Chapter 1

## Literature Review

### 1 | INTRODUCTION

Cultivated alfalfa (*Medicago sativa* ssp. *sativa*) is a major forage crop that plays a key role in global livestock production. Alfalfa hay is a relatively cheap, high-quality forage which is high in protein and thus an ideal feed for ruminant animals (Radović et al., 2009). Often referred to as the ‘Queen of forages’, alfalfa is one of California’s most important crops, driving production of the state’s most significant agricultural enterprise: dairy (Russelle, 2001; Zaccaria et al., 2017). In 2022, 200,000 hectares of alfalfa were harvested for hay and haylage with a total value of over \$1B (United States Department of Agriculture [USDA], 2023). When combined with the state’s \$10.4B value of milk production, alfalfa accounts for a significant portion of California’s agricultural GDP. In addition to its advantages as a forage, alfalfa also provides a host of beneficial ecosystem services: as a legume, alfalfa requires no nitrogen fertiliser, instead it fixes atmospheric nitrogen through a symbiotic relationship with rhizobia, a nitrogen fixing bacterium; the perennial nature of alfalfa improves soil health allowing fields to recover from frequent tilling and prevents topsoil loss; long roots can access water and nutrients deep in the soil profile and increase soil organic matter throughout; and alfalfa is an important insectary, hosting a diversity of beneficial insects (Putnam et al., 2007).

Yield is the most important trait for profitable alfalfa production, yet somewhat inexplicably, yield improvement in alfalfa has stalled over the last ~30 years (Wiersma et al., 1997; Volenec et al., 2002; Lamb et al., 2006; USDA, 2023). In addition to the lack of yield improvement and despite the significant economic and environmental benefits of alfalfa, the area cultivated has been in steady

decline both nationally and in California since a peak in 1960 (USDA, 2023). Already disadvantaged by the distribution of federal subsidies, of which commodity row crops receive billions each year, alfalfa faces stiff competition for inclusion in crop rotations. Increasing yield is therefore imperative to curb the rate of decline in alfalfa area, not only to continue to support California's significant livestock industry, but also for the benefits alfalfa provides to agricultural ecosystems.

Historically, breeders have relied on traditional breeding methods to increase biomass yield in alfalfa, with little success in recent years (Lamb et al., 2006). However, with the advent of new breeding technologies, such as genomic selection and high throughput phenotyping, this lack of yield gain may be approached in a new light. This PhD project investigates the incorporation of modern breeding tools and methodologies into an existing breeding program to address the lack of yield improvement in alfalfa.

## 2 | ORIGIN, SYSTEMATICS, AND BIOLOGY OF ALFALFA

Alfalfa is believed to have two centers of origin, Asia Minor/Caucasia (modern-day Iran, Armenia, Georgia, and Turkey) and Central Asia (Kazakhstan, Uzbekistan, and Afghanistan) (Wang & Şakiroğlu, 2021). Following domestication, alfalfa quickly spread throughout the ancient world due to its adaptability to diverse climates and soils. The expansion of civilizations and the Silk Road trade routes played a pivotal role in the dissemination of alfalfa, enabling its introduction to regions such as Europe, North Africa, and eventually the Americas.

Alfalfa exists naturally as the *Medicago sativa* complex that includes a range of diploid and autotetraploid subspecific taxa (Quiros & Bauchan, 1988). Cultivated alfalfa primarily refers to the subspecies *sativa* and is an autotetraploid ( $2n = 4x = 32$ ), perennial, outcrossing legume with

polysomic inheritance (Blondon et al., 1994). It has a basic chromosome number of eight and a genome size of 800-1000 Mb.

Commercial alfalfa stands typically last three to eight years depending on variety, soil, climate, and cultural practices. Pure stands are sown at high density with typical sowing rates ranging from 16-22 kg ha<sup>-1</sup> (Rankin, 2008). Plant density starts high with up to 800 plants m<sup>-2</sup> three months after planting and then steadily declines (Hall et al., 2004). In established alfalfa stands, plant density has limited effect on dry matter yield per hectare until plant numbers fall below 40 plants m<sup>-2</sup>, wherein yields decrease and growers should consider retiring the stand.

Alfalfa can grow to heights above one metre and has a deep root system that reaches beyond six metres in depth when grown in deep, well drained, moist soils (Johnson et al., 1998). Alfalfa plants generally have a single deep taproot, with variation in the number and size of lateral roots. Following establishment, alfalfa forms a crown at the top of the root system. After defoliation, alfalfa regrowth occurs from buds located on the crown and from axillary buds at nodes from the remaining above ground stubble (Pembleton et al., 2010). This regrowth cycle allows for multiple cuttings during the growing season for up to eight years before forage yields decline below economic thresholds.

Fall (or autumn) dormancy is an important characteristic of alfalfa that defines a population's fitness for specific agricultural environments. Fall dormancy is a plant's response to decreasing photoperiod and temperature and is associated with a slowing and eventual cessation of growth through the dormant period (Thomashow, 1999). This trait is closely related to the ability for populations to avoid winter kill and is under complex quantitative genetic control (Munjal et al., 2018). Alfalfa varieties are classified into different fall dormancy groups, typically ranging from 1 to 11, with lower numbers referring to dormant varieties and higher numbers representing reduced fall

dormancy. Non-dormant varieties tend to be higher yielding, but may be lower quality and less persistent, therefore appropriate variety selection is of key concern to the grower.

### 3 | ALFALFA PRODUCTION IN CALIFORNIA

Californian agricultural regions have a broad range of climates and soils and can be divided into five main growing regions: the Central Valley, Intermountain, Low Desert, High Desert, and Coastal regions (Putnam et al., 2007). Statewide average dry matter yields for alfalfa are 15-17 MG ha<sup>-1</sup> from an average of 6-7 harvests per year although this varies with up to 12 cuts on stands grown in the Low Desert and as few as 3 on fields in the Intermountain region (USDA 2023).

The Central Valley is the most significant area accounting for 70 percent of the state's alfalfa production (USDA, 2023). It has a Mediterranean climate characterized by hot, dry summers (average daily maximum temperatures of 38°C) and cool winters (average daily maximum temperatures of 16°C) (National Oceanic and Atmospheric Administration [NOAA], 2023). Rainfall totals range from 20-46 cm annually, falling predominantly in the cooler months from November through to March. The Central Valley has fertile, deep, alluvial soils, although some areas suffer from high salinity. Varieties grown in the Central Valley are predominantly semi-dormant to non-dormant (fall dormancy ratings between 6-10) however, in the northern tip of the valley dormant varieties (ratings from 3-4) are grown for better persistence in heavy soils and greater forage quality.

The Low Desert region in Southern California contains 17 percent of alfalfa production. An area with extremely low rainfall (less than 8 cm per year) and high temperatures (average summer daily maximum of 41°C, and winter daily maximum of 24°C) (NOAA, 2023; USDA, 2023). Non-dormant varieties are grown here with harvests occurring year-round. Soils are generally heavy and



like in the Central Valley, high salinity is a significant issue. The Low Desert is also an important alfalfa seed production area.

The final area of significance for alfalfa production is the Intermountain region in Northern California accounting for approximately 10 percent of production (USDA, 2023). This area is more temperate, located at high elevation with warm summers (average daily maximum of 30°C) and cool winters (average daily maximum of 7°C) (NOAA, 2023). Rainfall averages about 51 cm per year falling in the cooler months. Freezing winters necessitate the use of dormant cultivars to prevent winter kill.

Almost all the alfalfa grown in California is irrigated. Check-flood surface irrigation is the most common in the Central Valley and Low Desert regions, while sprinklers are the preferred method in the Intermountain area. Although a significant crop in California, the area of land cultivated in alfalfa has been in steady decline since a peak in 1960. Over the last 12 years hectareage has decreased by more than 40 percent from 390,000 ha in 2011 to 235,000 ha in 2022 (California Department of Food and Agriculture [CDFA], 2023). The predominant end-use of alfalfa grown in California is hay for dairy cattle. California surpassed Wisconsin as the number-one dairy state in 1993 and now produces more than 21 percent of milk in the United States (Putnam et al., 2007). At least 75 percent of alfalfa grown in California is used to supply the dairy industry.

#### 4 | ALFALFA BREEDING

The breeding goals for alfalfa are characteristic of most plant improvement programs. They include increasing yield, enhancing nutritive value, and improving biotic and abiotic stress tolerance (Lamb et al., 2006). The majority of desired traits are complex and quantitatively inherited; however, some pest and disease resistance mechanisms are likely under simple genetic control.

Alfalfa breeding programs are based on recurrent phenotypic selection, with or without progeny testing. They are designed to increase the frequency of desirable alleles for quantitatively inherited traits, while maintaining genetic variability for continued genetic improvement (Hallauer et al., 2010). Although self-fertilization is common, alfalfa suffers from severe inbreeding depression which is prohibitive to the production of hybrids, thus commercial cultivars are marketed as synthetic populations generated by crossing different numbers of selected genotypes (Jones & Bingham, 1995). As a consequence of the structure of the alfalfa genome, cross pollination and severe inbreeding depression, cultivars exhibit high levels of genetic variation (Nagl et al., 2011).

Significant gains have been made in most traits of interest in alfalfa. Forage quality has improved, demonstrated by the release of new high-quality cultivars (Hall et al., 2000; Lamb et al., 2006). Current cultivars exhibit resistance to a suite of pests and diseases including bacterial wilt (*Clavibacter michiganense* subsp. *insidiosum*), Verticillium wilt (*Verticillium albo-atrum*), Fusarium wilt (*Fusarium oxysporum* f.sp. *medicaginis*), anthracnose (*Colletotrichum trifolii*), Phytophthora root rot (*Phytophthora medicaginis*), Aphanomyces root rot (*Aphanomyces euteiches*), root-knot nematodes (*Meloidogyne* spp.), stem nematode (*Ditylenchus dipsaci*), spotted alfalfa aphid (*Therioaphis maculata*), pea aphid (*Acyrtosiphon pisum*), and others (Li & Brummer, 2012; National Alfalfa and Forage Alliance [NAFA], 2023). However, there has been little to no improvement in yield, for which a variety of explanations have been proposed. Perennial forage breeders are at a disadvantage compared to those working with annual grain crops when it comes to yield improvement due in part to its perennial nature requiring multiple years of evaluation before selection can be made, the negative genetic correlation between forage quality and forage yield, and the inability to make gains in the harvest index that is possible in grain crops, as all above ground biomass is harvested (Lamb et al., 2006). A lack of forage breeders and limited resources constrain the size and scope of breeding trials, reducing their efficacy in improving traits with low heritability, such as yield. The emphasis by alfalfa

breeders on pest and disease resistance and greater persistence may lead to a realization of yield potential but it is not increasing yield *per se*. Perhaps the most important reason for the lack of yield improvement in alfalfa is that breeders have not been explicitly selecting for increased yield potential under commercial production conditions. Yield is often selected indirectly based on evaluation of vigor on spaced plants or on short family rows rather than on measurements of yield on plots grown as a densely sown sward (Brummer & Casler, 2014).

Although little can be done to address issues such as the inability to increase the harvest index of alfalfa, we do have the ability to modify our trial methodology and to utilize modern technology such as genomic selection and high throughput phenotyping to improve yield potential in alfalfa. Measuring yield on plots instead of individual plants provides a better proxy of commercial production systems, and the incorporation of remote sensing, high throughput phenotyping and genomic selection allow for better allocation of resources within a breeding program and can help to speed up the rate of genetic gain through shorter selection cycles.

## 5 | GENOMIC SELECTION

Genomic selection (GS) is a form of marker-assisted selection (MAS) where the breeding value for a given trait of a genotype can be estimated from many markers distributed across the entire genome (Meuwissen et al., 2001). MAS is based upon the establishment of a tight linkage between a molecular marker and the chromosomal location of the gene(s) governing the trait to be selected in a particular environment (Talukdar, 2013). This is useful when working with traits controlled by a few large-effect loci, however this is not the case for many important traits in plant breeding, including biomass yield. GS expands on this idea and guides selection based on the cumulative impact of many small-effect loci that are in linkage disequilibrium (LD) with genetic markers (Goddard &

Hayes, 2009). To estimate breeding values for a genotype, first a model must be developed from a training population that exhibits variation for the trait of interest. This training population is both genotyped and phenotyped to develop a model which optimizes the chromosomal pattern of alleles. This model then allows breeders to estimate the genetic potential of unobserved genotypes based solely on marker information. Because it relies only on a plant's genotype, which can be determined when the plant is still very young, genomic selection allows significantly shorter selection cycles than are required under recurrent phenotypic selection, thus has the potential to significantly increase the rate of genetic gain in an alfalfa breeding program.

Early methods of GS in plant breeding used gene chip arrays and were mostly adopted by well-resourced breeding programs in major crop species (corn, wheat, rice, etc.) (Crosa et al., 2017). As high-throughput sequencing costs have decreased, sequencing based approaches have become more viable and GS is now being explored in a wide range of crops. Genotyping-by-sequencing (GBS) is currently the most popular reduced-representation approach for genomic selection (Hawkins & Yu, 2018). It is a reduced-representation approach that uses restriction enzymes to cut the genome at fixed points to show good overall coverage. Samples are sequenced using high-throughput next-generation sequencing platforms. A variety of software programs (e.g. TASSEL and NGSEP 3)) can then be used for genotype calling and identification of polymorphisms for downstream analysis (Bradbury et al., 2007; Tello et al., 2019). Because genome coverage is incomplete, causal loci are unlikely to be identified, but the polymorphisms are likely to be in linkage disequilibrium (LD) with loci that are causal. Consequently, recombination between the causal and marker loci will occur during the breeding process and as allele frequencies change with each selection cycle, LD shifts impacting the accuracy of predictive models over time. GS models therefore require regular updating and as such, model training becomes an important component of a modern breeding system. With the cost of genotyping rapidly decreasing and the recent release of

multiple chromosome-scale, haplotype-phased genome assemblies for alfalfa (Chen et al., 2020; Shen et al., 2020; Long et al., 2022), genomics is becoming a viable option for many smaller breeding programs.

Recently the use of GS has been investigated by alfalfa breeders for biomass yield (Annicchiarico et al., 2015; Li et al., 2015), forage quality (Biazzi et al., 2017) and salinity tolerance (Medina et al., 2020). However, these studies were based on phenotypic and/or genotypic data at the individual plant level. Although useful, alfalfa is often evaluated at the family level using half- or full-sib families and then marketed as a synthetic population. Andrade et al. (2022) proposed GS may be better incorporated into an alfalfa breeding program by genotyping pooled families to obtain allele frequency marker data rather than individual genotyping calls.

One major takeaway from much of the work in GS is the size of the training population plays a key role in the predictive ability of the final model (de Bem Oliveira et al., 2020). However, for lesser funded breeding programs, genotyping and phenotyping can quickly become prohibitively expensive with the inclusion of more material. Pooled genotyping is one method of lowering the cost to breeders. Another is incorporating remote sensing and high throughput phenotyping to reduce the expensive labour component of phenotyping the training population.

## 6 | HIGH-THROUGHPUT PHENOTYPING AND REMOTE SENSING

Plant phenotyping is a core foundation of plant breeding and has evolved through the years. Accurate and rapid measurement of phenotypic data is essential to understanding the genetic basis for plant traits and for the subsequent generation of improved germplasm. For biomass yield in alfalfa this traditionally required the destructive sampling, drying and weighing of hundreds to thousands of experimental units multiple times over the 2-4 year lifespan of alfalfa breeding trials.

This process is labor-intensive, time-consuming, and costly. Recently, improvements in camera technology, aerial photography, and data processing have resulted in the broad adoption of remote sensing (RS) and high throughput phenotyping (HTP) in agriculture which can significantly reduce the high labor cost of phenotyping.

Remote sensing allows for the accurate, efficient, and non-destructive estimate of biomass and has been shown to be useful for high throughput phenotyping in breeding applications (Araus & Cairns, 2014; Tattaris et al., 2016), including the prediction of biomass yield in large alfalfa breeding plots (Feng et al., 2020). What is not yet clear however, is whether the same predictive ability transfers to the variety of other plot types used in alfalfa breeding; family rows and mini-sward plots. This is of particular interest for training a genomic selection model where upwards of 1000 families need to be evaluated for optimal predictive ability (de Bem Oliveira et al., 2020).

The benefits of successfully incorporating HTP into an alfalfa breeding program will be two-fold. Firstly, it will enable trial sizes to increase, benefitting not only training populations for genomic selection, allowing greater prediction accuracies, but will be useful to upscale traditional evaluation trials. Secondly, non-destructive biomass measurement will allow the tracking of growth rates throughout the season and other temporal traits, something that is not currently feasible with traditional destructive harvest methods.

From the review of the literature, the following conclusions can be made:

- Alfalfa is a significant contributor to the Californian agricultural GDP and a core constituent in the diet of the nation's largest dairy industry. It therefore warrants further plant breeding attention to continue improving its economic traits.
- Beyond its role as a forage, alfalfa provides a host of additional benefits including: its role in crop rotations for its nitrogen fixation capabilities and improved soil health, its perenniality resulting in reduced tillage and topsoil loss and as a habitat for native fauna.
- Continued production of alfalfa in California is under threat with cultivated area in steady decline since the 1960s. In addition, yield improvement has stagnated over the past 30 years. Modern breeding methodologies may help to address the lack of yield improvement and keep it as a viable option in crop rotations.
- The cost of genomics is rapidly decreasing and genomic tools such as reference genomes and analysis software are now readily available for smaller breeding programs.
- Genomic selection has proven to be successful in increasing the rate of genetic gain in other crops and has been useful in alfalfa at the individual plant level. It is unclear however, whether this will translate to an increase in yield potential, therefore investigation of GS using family bulks is warranted.
- Remote sensing and high throughput phenotyping methods have greatly improved in recent years and are now widely utilized in agricultural research. They have proven useful in assessing biomass yield in alfalfa at the large plot level, but further research is required to see if this translates to other breeding plot types.

The objectives of experiments in this thesis were to increase the overall yield potential of alfalfa through the development of new populations using genomic- and phenomic-based breeding.

Five objectives were identified, to:

1. Analyze results from a yield evaluation trial established in 2017 to compare conventional phenotypic selection methods with genomic selection based on a model developed from individual plant phenotypes.
2. Establish a multi-site transplanted half-sib family trial with mini sward plots to evaluate biomass yield in elite UC Davis alfalfa germplasm.
3. Develop an allele frequency-based genomic prediction model using family bulks to calculate genomic estimated breeding values (GEBVs) for biomass yield.
4. Make selections and intercross selected genotypes using conventional phenotypic selection and genomic selection to develop new populations to be assessed in future yield evaluation trials.
5. Assess the predictive ability of remote sensing and high throughput phenotyping for biomass yield across a range of plot types commonly used in alfalfa breeding trials.



- Andrade, M. H., Acharya, J. P., Benevenuto, J., de Bem Oliveira, I., Lopez, Y., Munoz, P., . . . Rios, E. F. (2022). Genomic prediction for canopy height and dry matter yield in alfalfa using family bulks. *The Plant Genome*, *15*. doi:<https://doi.org/10.1002/tpg2.20235>
- Annicchiarico, P., Nazzicari, N., Li, X., Wei, Y., Pecetti, L., & Brummer, E. C. (2015). Accuracy of genomic selection for alfalfa biomass yield in different reference populations. *BMC Genomics*, *16*, 1020. doi:<https://doi.org/10.1186/s12864-015-2212-y>
- Araus, J. L., & Cairns, J. E. (2014). Field high-throughput phenotyping: the new crop breeding frontier. *Trends in Plant Science*, *19*(1), 52-61.  
doi:<https://doi.org/10.1016/j.tplants.2013.09.008>
- Biazzi, E., Nazzicari, N., Pecetti, L., Brummer, E. C., Palmonari, A., Tava, A., & Annicchiarico, P. (2017). Genome-wide association mapping and genomic selection for alfalfa (*Medicago sativa*) forage quality traits. *PLoS ONE*, *12*(1).  
doi:<https://doi.org/10.1371/journal.pone.0169234>
- Blondon, F., Marie, D., Brown, S., & Kondorosi, A. (1994). Genome size and base composition in *Medicago sativa* and *M. truncatula* species. *Genome*, *37*(2), 264-270. doi:10.1139/g94-037.  
PMID: 18470076.
- Brummer, E. C., & Casler, M. D. (2014). Cool-season forages. In S. Smith, B. Diers, J. Specht, & B. Carver (Eds.), *Yield Gains in Major U.S. Field Crops* (pp. 33-52). Madison, WI: ASA, CSSA, SSSA.
- California Department of Food and Agriculture (CDFA). (2023). Field Crops. In *California Agricultural Statistics Review 2021-2022*.  
doi:[https://www.cdfa.ca.gov/Statistics/PDFs/2022\\_Ag\\_Stats\\_Review.pdf](https://www.cdfa.ca.gov/Statistics/PDFs/2022_Ag_Stats_Review.pdf)

- Chen, H., Zeng, Y., Yang, Y., Huang, L., Tang, B., Zhang, H., . . . Qiu, Q. (2020). Allele-aware chromosome-level genome assembly and efficient transgene-free genome editing for the autotetraploid cultivated alfalfa. *Nature Communications*, *11*, 2494.  
doi:<https://doi.org/10.1038/s41467-020-16338-x>
- Crossa, J., Pérez-Rodríguez, P., Cuevas, J., Montesinos-López, O., Jarquín, D., de los Campos, G., . . . Varshney, R. K. (2017). Genomic selection in plant breeding: methods, models and perspectives. *Trends in Plant Science*, *22*(11), 961-975.  
doi:<https://doi.org/10.1016/j.tplants.2017.08.011>
- de Bem Oliveira, I., Amadeu, R. R., Ferrão, L. F., & Muñoz, P. R. (2020). Optimizing whole-genomic prediction for autotetraploid blueberry breeding. *Heredity*, *125*, 437-448.  
doi:<https://doi.org/10.1038/s41437-020-00357-x>
- Feng, L., Zhang, Z., Ma, Y., Du, Q., Williams, P., Drewry, J., & Luck, B. (2020). Alfalfa yield prediction using UAV-based hyperspectral imagery and ensemble learning. *Remote Sensing*, *12*(12), 2028. doi:<https://doi.org/10.3390/rs12122028>
- Goddard, M., & Hayes, B. (2009). Mapping genes for complex traits in domestic animals and their use in breeding programmes. *Nature Reviews Genetics*, *10*, 381-391.  
doi:<https://doi.org/10.1038/nrg2575>
- Hall, M. H., Nelson, C. J., Coutts, J. H., & Stout, R. C. (2004). Effect of seeding rate on alfalfa stand longevity. *Agronomy Journal*, *96*, 717-722. doi:<https://doi.org/10.2134/agronj2004.0717>
- Hall, M. H., Smiles, W. S., & Dickerson, R. A. (2000). Morphological development of alfalfa cultivars selected for higher quality. *Agronomy Journal*, *92*, 1077-1080.  
doi:<https://doi.org/10.2134/agronj2000.9261077x>

- Hallauer, A. R., Filho, J. B., & Carena, M. J. (2010). Breeding plans. In *Quantitative Genetics in Maize Breeding* (Vol. 6). New York, NY: Springer. doi:[https://doi.org/10.1007/978-1-4419-0766-0\\_12](https://doi.org/10.1007/978-1-4419-0766-0_12)
- Hawkins, C., & Yu, L. (2018). Recent progress in alfalfa (*Medicago sativa* L.) genomics and genomic selection. *The Crop Journal*, 6(6), 565-575. doi:<https://doi.org/10.1016/j.cj.2018.01.006>
- Johnson, L. D., Marquez-Ortiz, J. J., Lamb, J. F., & Barnes, D. K. (1998). Root morphology of alfalfa plant introductions and cultivars. *Crop Science*, 38, 497-502. doi:<https://doi.org/10.2135/cropsci1998.0011183X003800020037x>
- Jones, J. S., & Bingham, E. T. (1995). Inbreeding depression in alfalfa and cross-pollinated crops. In J. Janick (Ed.), *Plant Breeding Reviews* (pp. 209-233). John Wiley & Sons. doi:<https://doi.org/10.1002/9780470650059.ch6>
- Lamb, J. F., Sheaffer, C. C., Rhodes, L. H., Sulc, R. M., Undersander, D. J., & Brummer, E. C. (2006). Five decades of alfalfa cultivar improvement: impact on forage yield, persistence, and nutritive value. *Crop Science*, 46, 902-909. doi:<https://doi.org/10.2135/cropsci2005.08-0236>
- Li, X., & Brummer, E. C. (2012). Applied genetics and genomics of alfalfa breeding. *Agronomy*, 2, 40-61. doi:<https://doi.org/10.3390/agronomy2010040>
- Li, X., Wei, Y., Acharya, A., Hansen, J. L., Crawford, J. L., Viands, D. R., . . . Brummer, E. C. (2015). Genomic prediction of biomass yield in two selection cycles of a tetraploid alfalfa breeding population. *The Plant Genome*, 8. doi:<https://doi.org/10.3835/plantgenome2014.12.0090>
- Long, R., Zhang, F., Zhang, Z., Li, M., Chen, L., Wang, X., . . . Yang, Q. (2022). Genome assembly of alfalfa cultivar Zhongmu-4 and identification of SNPs associated with agronomic traits. *Genomics, Proteomics & Bioinformatics*, 20(1), 14-28. doi:<https://doi.org/10.1016/j.gpb.2022.01.002>

- Medina, C. A., Hawkins, C., Liu, X., Peel, M., & Yu, L. (2020). Genome-wide association and prediction of traits related to salt tolerance in autotetraploid alfalfa (*Medicago sativa* L.). *International Journal of Molecular Sciences*, *21*(9), 3361.  
doi:<https://doi.org/10.3390/ijms21093361>
- Meuwissen, T. H., Hayes, B. J., & Goddard, M. E. (2001). Prediction of total genetic value using genome-wide dense marker maps. *Genetics*, *157*(4), 1819-1829.  
doi:<https://doi.org/10.1093/genetics/157.4.1819>
- Munjal, G., Hao, J., Teuber, L. R., & Brummer, E. C. (2018). Selection mapping identifies loci underpinning autumn dormancy in alfalfa (*Medicago sativa*). *G3*, *8*(2), 461-468.  
doi:[10.1534/g3.117.300099](https://doi.org/10.1534/g3.117.300099)
- Nagl, N., Taski-Ajdukovic, K., Barac, G., Baburski, A., Seccareccia, I., Milic, D., & Katic, S. (2011). Estimation of the genetic diversity in tetraploid alfalfa populations based on RAPD markers for breeding purposes. *International Journal of Molecular Sciences*, *12*(8), 5449-5460.  
doi:<https://doi.org/10.3390/ijms12085449>
- National Alfalfa and Forage Alliance (NAFA). (2023). *Alfalfa Variety Ratings*.  
doi:[https://www.alfalfa.org/pdf/2023\\_Alfalfa\\_Variety\\_Leaflet.pdf](https://www.alfalfa.org/pdf/2023_Alfalfa_Variety_Leaflet.pdf)
- National Oceanic and Atmospheric Administration (NOAA). (2023). Climate Monitoring.  
doi:<https://www.nci.noaa.gov/access/monitoring/products/>
- Pembleton, K. G., Volenec, J. J., Rawnsley, R. P., & Donaghy, D. J. (2010). Partitioning of taproot constituents and crown bud development are affected by water deficit in regrowing alfalfa. *Crop Science*, *50*, 989-999. doi:<https://doi.org/10.2135/cropsci2009.03.0140>
- Putnam, D. H., Orloff, S. B., & Teuber, L. R. (2007). Choosing an alfalfa variety. In C. G. Summers, & D. H. Putnam (Eds.), *Irrigated Alfalfa Management for Mediterranean and Desert Zones* (pp. 59-72). Oakland: University of California Agriculture and Natural Resources.

- Putnam, D. H., Summers, C. G., & Orloff, S. B. (2007). Alfalfa production systems in California. In C. G. Summers, & D. H. Putnam (Eds.), *Irrigated Alfalfa Management for Mediterranean and Desert Zones* (pp. 1-18). Oakland: University of California Agriculture and Natural Resources Publication 8287. doi:<http://alfalfa.ucdavis.edu/IrrigatedAlfalfa>
- Quiros, C. F., & Bauchan, G. R. (1988). The genus *Medicago* and the origin of the *Medicago sativa* comp. In A. A. Hanson, D. K. Barnes, & R. R. Hill (Eds.), *Alfalfa and Alfalfa Improvement*. American Society of Agronomy. doi:<https://doi.org/10.2134/agronmonogr29.c3>
- Radović, J., Sokolović, D., & Marković, J. (2009). Alfalfa—most important perennial forage legume in animal husbandry. *Biotechnology in Animal Husbandry*, 25(5-6-1), 465-475.
- Rankin, M. (2008). Determining the optimum alfalfa seeding rate. *Focus on Forage*, 10(5), 1-2.
- Russelle, M. P. (2001). Alfalfa: After an 8,000-year journey, the “Queen of Forages” stands poised to enjoy renewed popularity. *American Scientist*, 89(3), 252-261.  
doi:<http://www.jstor.org/stable/27857472>
- Shen, C., Du, H., Chen, Z., Lu, H., Zhu, F., Chen, H., . . . Wang, T. (2020). The chromosome-level genome sequence of the autotetraploid alfalfa and resequencing of core germplasms provide genomic resources for alfalfa research. *Molecular Plant*, 13, 1250-1261.  
doi:<https://doi.org/10.1016/j.molp.2020.07.003>
- Talukdar, D. (2013). Leguminosae. In S. Maloy, & K. Hughes (Eds.), *Brenner's Encyclopedia of Genetics (Second Edition)* (pp. 212-216). Academic Press. doi:<https://doi.org/10.1016/B978-0-12-374984-0.00854-8>
- Tattaris, M., Reynolds, M. P., & Chapman, S. C. (2016). A direct comparison of remote sensing applications for high-throughput phenotyping in plant breeding. *Frontiers in Plant Science*, 7.  
doi:<https://doi.org/10.3389/fpls.2016.01131>
- Tello, D., Gil, J., Loaiza, C. D., Riascos, J. J.,

- Cardozo, N., & Duitama, J. (2019). NGSEP3: accurate variant calling across species and sequencing protocols. *Bioinformatics*, 35(22). <http://doi.org/10.1093/bioinformatics/btz275>
- Thomashow, M. F. (1999). Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. *Annual Review of Plant Physiology and Plant Molecular Biology*, 50(1), 571-599. doi:<https://doi.org/10.1146/annurev.arplant.50.1.571>
- United States Department of Agriculture (USDA). (2023). National Agricultural Statistics Service Quick Stats. doi:<https://quickstats.nass.usda.gov/>
- Volenc, J. J., Cunningham, S. M., Haagenson, D. M., Berg, W. K., Joern, B. C., & Wiersma, D. W. (2002). Physiological genetics of alfalfa improvement: past failures, future prospects. *Field Crops Research*, 75(2-3), 97-110. doi:[https://doi.org/10.1016/S0378-4290\(02\)00020-5](https://doi.org/10.1016/S0378-4290(02)00020-5)
- Wang, Z., & Şakiroğlu, M. (2021). The origin, evolution and genetic diversity of alfalfa. In L.-X. Yu, & C. Kole (Eds.), *The Alfalfa Genome* (pp. 29-31). Springer Cham. doi:<https://doi.org/10.1007/978-3-030-74466-3>
- Wiersma, D., Undersander, D., Lauer, J., & Grau, C. (1997). Lack of alfalfa yield progress in the Midwest. *Proceedings of the Central Alfalfa Improvement Conference*, 36.
- Zaccaria, D., Carrillo-Cobo, M. T., Montazar, A., Putnam, D. H., & Bali, K. (2017). Assessing the viability of sub-surface drip irrigation for resource-efficient alfalfa production in Central and Southern California. *Water*, 9(11), 837. doi:<https://doi.org/10.3390/w9110837>

# Chapter 2

## An Empirical Test of Genomic Selection for Forage Dry Matter Yield in Alfalfa

### 1 | INTRODUCTION

Alfalfa (*Medicago sativa* L. subsp. *sativa*) is one of the most widely grown perennial forage legumes in temperate and Mediterranean-climate regions worldwide (Annicchiarico et al., 2015), owing to its exceptional yield, high nutrition, broad adaptability, nitrogen fixation, and host of beneficial ecosystem services (Putnam et al., 2007). Alfalfa hay grown in California predominantly supports the largest dairy sector in the USA, but also provides forage for sheep, beef, and horse production as well as a growing export market (Martin & Mertins, 2005). Alfalfa is an allogamous autotetraploid ( $2n = 4x = 32$ ) and is characterized by severe inbreeding depression (Li & Brummer, 2009). Alfalfa is highly heterozygous, and cultivars are synthetic populations that exhibit high variability (Annicchiarico & Pecetti, 2021). Most breeding programs currently utilize recurrent phenotypic selection, where the best genotypes are recombined following evaluation trials that typically last 2-4 years (Li et al., 2015; Andrade et al., 2022).

Despite the numerous benefits of alfalfa, the economic viability of alfalfa is under threat from an increasing yield gap relative to major cereal crops and other potential substitutes in the dairy ration (Annicchiarico et al., 2015). This yield gap has developed due to low rates of genetic gain for forage yield in alfalfa, particularly over the last 30 years where progress has stalled completely (Brummer & Casler, 2014). This lack of yield improvement can be ascribed to a range of factors common in outcrossing perennial forages, namely long selection cycles, multiple harvests per year,

small breeding investment, the inability to develop hybrids, the harvesting of all above ground biomass (therefore an inability to alter the harvest index), the need to maintain forage nutritive value, and significant genotype by environment interaction (G x E) (Li & Brummer, 2012; Annicchiarico, 2015). However, yield improvement has occurred in other perennial forages such as perennial ryegrass (*Lolium perenne*) (McDonagh et al., 2016) and white clover (*Trifolium repens* L.) (Woodfield & Caradus, 1994; Hoyos-Villegas, 2019); therefore, progress should be possible in alfalfa. Lamb et al. (2006) suggested that the lack of yield improvement in alfalfa is because less breeding focus has been placed on yield, instead there has been a focus on improving tolerance to biotic and abiotic stresses. Although this enables alfalfa to reach its yield potential, it is not increasing yield *per se* in populations under improvement. Furthermore, alfalfa yield is often selected indirectly based on evaluation of vigor on spaced plants or on family rows (Li & Brummer, 2012), which has been shown to be a poor proxy for forage yield in the dense swards used in commercial alfalfa production (Annicchiarico, 2006).

Marker-assisted selection (MAS) is a useful tool for plant breeding programs and may be one way to improve the rate of genetic gain. Early research enabled breeders to identify molecular markers strongly linked to quantitative trait loci (QTL) for a variety of important traits in alfalfa (McCord et al., 2014). However, MAS is primarily effective for traits controlled by relatively few genes with large effects. Complex traits, including yield, are usually controlled by many loci with small effects (Medina et al., 2021). In this case, genomic selection (GS) offers a compelling alternative to MAS by using a model that includes the effect of all markers in computing a genomic estimated breeding value (GEBV) for each individual in the population. Genomic selection can address one of the largest impediments to faster genetic gain in alfalfa – the need for multi-year evaluations that extend the length of each selection cycle. Selection can be made on genotypic information alone without the need for phenotypic evaluation, reducing the cycle time length from



3-5 years to less than 6 months. With the cost of high-throughput sequencing decreasing and the recent publication of multiple chromosome-scale, haplotype phased genome assemblies for tetraploid alfalfa (Chen et al., 2020; Shen et al., 2020), the prospect of a robust genomic selection program is now possible for many alfalfa breeding programs.

Various studies have investigated the use of GS in alfalfa breeding for a range of traits including biomass yield, forage quality and salinity tolerance. Moderate prediction accuracies were obtained for biomass yield, stem digestible neutral detergent fiber (NDFD), and leaf protein content, ranging from 0.3-0.4 (Annicchiarico et al., 2015; Biazzi et al., 2017). The vigor of alfalfa under salt stress has also been assessed and a predictive model developed with a prediction accuracy of 0.793 (Medina et al., 2020). Although the results of these studies suggest GS could be used to increase the rate of genetic gain for a range of traits in alfalfa, no empirical demonstration of GS has been published to date.

We hypothesized that using genomic selection for high yield based on a model developed from the phenotypic evaluation of clonally replicated genotypes would result in higher yield than a population selected by genomic selection for low yield or than phenotypic selection. The objective of this study was to empirically test populations developed from a genomic selection model for forage dry matter yield in densely sown sward plots of alfalfa.

## 2 | MATERIALS AND METHODS

### 2.1 | Germplasm development

The germplasm used for genomic selection derived from a population created by Dr. Don Viands at Cornell Univ. in the 1990s called NY0358. We previously described this population, the NE-1010 clonal selection population, in an experiment using SSR markers for association analysis (Li et al.,

2011a). Briefly, NY0358 was formed by intercrossing three elite, semi-dormant cultivars and recombining the resulting population twice.

The NY0358 population underwent two cycles of selection for biomass yield using clonal evaluations at multiple locations (Figure 1). About 200 individual plants (aka “genotypes”) were included at the beginning of each cycle. These plants were clonally propagated using stem cuttings, and three replications were planted to the field at each location. In each replication, three clones were included in a plot; thus, each individual genotype was replicated nine times at each location in each cycle. Yield data were collected across multiple harvests and multiple years on individual plots, bulking the biomass of the three clones within the plot. In the first cycle, data were obtained from Ithaca, New York; Ste.-Foy, Québec; and Ames, Iowa. The top yielding 10% of genotypes selected based on an across location analysis of total annual yield were recombined to form NY0847. A second cycle of phenotypic selection was conducted using NY0847; genotypes were clonally propagated as for Cycle 1 and yield data collected from Ithaca, NY and Ste.-Foy, Québec. The best 10% of genotypes from NY0847 based on a statistical analysis of total annual biomass yield from NY only were intercrossed to form NY1221 (Li et al. 2015).

## 2.2 | Genomic selection

For genomic selection, we used a model developed from the initial clonal evaluation cycle (i.e., of NY0358) total annual yield measured in NY only, because these data were more robust than those from the other locations (Li et al. 2015). We based the model on total annual yield, which is a more important trait than yield of any individual harvest.

We applied the model to seedlings from the population NY0847 and subsequently conducted a second cycle of genomic selection. We grew 19 or 20 individual seedlings from each of the 20 maternal families composited to create NY0847, for a total of 384 individual seedlings

genotyped using GBS, as described previously (Li et al., 2015), multiplexing 100 genotypes in a single lane of a HiSeq 2000 DNA sequencer. We aligned sequences with previously determined sequence tags to only analyze SNP that had been part of the model (Li et al., 2015). Following SNP scoring and imputation, we computed GEBVs for each individual plant. Based on GEBVs, we selected the top 20 genotypes, restricting selections to no more than four individuals from any given maternal half-sib family to maintain variation in the population. These 20 individuals were intermated in the greenhouse by hand without emasculation to form the GSC1H population. An analogous population, GSC1L, based on the lowest 20 GEBVs was also formed. In addition, a random selection of 20 plants from the 400 plant population was intermated as a control population, GSC1R.

For the second cycle of selection, seeds of each maternal half-sib family used to form GSC1H were germinated and DNA from 19 or 20 plants from each of the 20 families was isolated for a total of 384 plants analyzed with GBS markers. We again selected the top and bottom 20 individuals based on GEBVs as done for Cycle 1 and intercrossed them separately in the greenhouse to create GSC2H and GSC2L, respectively.

### 2.3 | Experimental populations

We produced seed of all populations shown in Fig. 1 in 2016; the seed of the newly developed populations from phenotypic selection (PS) or GS is the Syn 2 generation; populations NY0358 and NY0847 are advanced generations beyond Syn 2. One gram of seed was sent to Mr. Chuck Burnett, a commercial seed producer in Idaho, who planted the seed of each entry in a single cage, enabling pollination by leaf cutter bees (*Megachile rotundata*). Seed was returned in autumn 2016 for planting in 2017.

## 2.3 | Yield Trials

Experiments were established in April 2017 at two locations in the United States each consisting of ten replications laid out in a randomized complete block design. The two locations were the Cornell University Research Farm in Ithaca, NY (42.45° N, 76.46° W) on a Niagara silt loam (fine-silty, mixed, active, mesic Aeric Endoaqualf) and 973 mm average annual rainfall); and Tulelake, CA (41°96' N, 121°47' W; 292 mm average annual rainfall), on a Tulebasin mucky silty clay loam (fine, mixed, mesic Aquandic Haplaquolls). The sowing rate was 20 kg ha<sup>-1</sup> with plots measuring approximately 1.5m × 5m. Each plot contained 8 rows spaced 17cm apart. An alfalfa border was sown around the entire experimental plot area. Soil tests were conducted at each location and fertilizer applied to maintain P and K at recommended levels for high yielding perennial forages (Meyer et al., 2007). Trials were monitored for weeds, insects, and mammalian pests, with control measures conducted accordingly.

Forage yields were estimated by mowing a swath through each plot leaving a residual of 7 cm. Prior to harvest, alleyways between plots were mown to remove edge effects and ensure plots were of uniform length. The target maturity for harvest was bud to early flowering stage. In Ithaca the harvest area was 1- by 4m. There was a total of nine harvests, three in each of 2018, 2019 and 2020, with no data collected in the establishment year. In Tulelake the harvest area was 0.9- by 4-m. There was a total of 12 harvests, three in 2017, four in 2018 and 2019, and a single harvest in 2020. Hand grab samples (~300 g wet weight) were collected and weighed from 20% of the plots at each harvest. They were dried in a forced-air drying oven at 55°C for seven days, reweighed and used to calculate dry matter percentage. All yields were recorded on a dry matter basis (kg DM ha<sup>-1</sup>), adjusting plot weights by the average dry matter percentage.

## 2.4 | Fall height trial

An experiment was established in April 2018 consisting of four replications laid out in a randomized complete block design. This trial was a large dormancy evaluation but included all populations in the yield trial described above except for the cycle one phenotypic selection population - NY1221. The trial was located in Davis, CA (38°52' N, 121°77' W; 497 mm average annual rainfall) on a Yolo silty clay loam (fine-silty, mixed, superactive, thermic Fluventic Haploxerepts). Plants were sown in trays in the greenhouse 2 months prior to transplanting. Plots consisted of a single row of 25 plants spaced 30 cm apart with a 90cm gap between plots within rows and 60cm spacing between rows. Plants were harvested throughout the season when they reached the target maturity of bud to early flowering stage using a self-propelled forage harvester. Fertilizer was applied to maintain P and K at appropriate levels for high yielding perennial forages, with weeds, insects and other pests monitored and standard control measures applied if necessary.

Plant height was measured 25 days after the final harvest in October in 2018 and 2019. Plant height was considered the distance from the soil surface to the tallest point of the plant at its natural height (without manually lifting any stems). A single measurement was taken from each plant and an average height across all plants in the plot was determined.

## 2.5 | Statistical analyses

All analyses were performed using R statistical software (v4.1.0; R Core Team 2021). For the yield trial, analysis of variance (ANOVA) was conducted to estimate the effects of population, location, and harvest on forage yield. Locations were analyzed independently followed by a multi-environment analysis. In addition to an overall annual yield analysis, the yield of individual harvests within location was conducted and across locations, harvests were analyzed by season, with spring (one harvest at both locations), summer (one harvest in New York; two harvests in California), and

fall (one harvest at both locations) groups included. For the single location models, population, block, harvest, and population  $\times$  block interaction were treated as fixed effects (Gezan, 2023). For the multi-environment models, population, location, harvest within location, block within location, population  $\times$  location interaction, and population  $\times$  harvest within location were treated as fixed effects. Means and standard errors were calculated on a per harvest basis for each population using the emmeans package (Lenth, 2021). Pairwise comparisons were conducted using the multcomp package (Hothorn et al., 2008). The significance threshold was set at 0.05 using the Tukey method for multiple comparisons.

Broad-sense heritability was calculated for each individual harvest and across all harvests within location and overall, across all harvests and locations. ASReml-R was used to fit linear mixed models using residual maximum likelihood for each harvest and overall, within and across locations (Butler, 2022). Variance components from the resulting equations were used to estimate population-based broad-sense heritability ( $H^2$ ) for individual harvests as:

$$H^2 = \frac{\sigma_g^2}{\sigma_P^2} = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_e^2}{r}},$$

where  $\sigma_P^2$  is the phenotypic variance,  $\sigma_g^2$  is the population variance and  $r$  is the number of reps.  $H^2$  for all harvests at a single location was estimated by:

$$H^2 = \frac{\sigma_g^2}{\sigma_P^2} = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_{gc}^2}{c} + \frac{\sigma_e^2}{c * r}},$$

where  $\sigma_{gc}^2$  is the variance for population by harvest interaction and  $c$  is the number of harvests.

Overall  $H^2$  was calculated as:

$$H^2 = \frac{\sigma_g^2}{\sigma_P^2} = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_{gl}^2}{l} + \frac{\sigma_{gc}^2}{c} + \frac{\sigma_e^2}{l * c * r}},$$

where  $\sigma_{gl}^2$  is the variance for population by location and  $l$  is the number of locations.

For fall height trial, ANOVA was used to estimate the effect of year and population on fall height. Population, year, block, and population  $\times$  year interaction were treated as fixed effects. Means, standard errors and pairwise comparisons were computed for fall height for each population using the same method as forage yield above.

### 3 | RESULTS

Forage yield differed among populations at each location and in the overall analysis (Table 1). Forage yield also differed among populations for the different seasonal harvests at each location. The base population (NY0847), phenotypic selection cycle one (PSC1 = NY1221), genomic selection cycle one – high (GSC1-H), and genomic selection cycle one – random (GSC1-R) were the top performing populations overall across both locations; the genomic selection cycle one – low (GSC1-L) and genomic selection cycle two populations (GSC2-H and GSC2-L) were the poorest performing. These trends were also observed in the seasonal breakdown of harvests, with the exception of GSC1-L which performed well in the autumn harvests. In Ithaca, NY0847, GSC1-H, and GSC1-R had the highest yield per harvest over the four-year duration of the trial. GSC1-L had low yield in the spring harvests and overall. GSC2-H and GSC2-L were consistently the lowest yielding populations. Although NY1221 was not significantly different to top performing varieties in the seasonal breakdown of harvests, the GSC1-H population had a significantly higher forage yield than NY1221 (PSC1) overall. In Tulelake, there was less separation between populations. NY0847 and GSC1-H yielded significantly more biomass than GSC1-L in spring, summer and overall; however GSC1-L outperformed GSC1-H in the fall. There was no significant difference between genomic selection and phenotypic selection in Tulelake.

Fall height was relatively consistent among populations in the Davis experiment (Figure 1). The only significant difference was that GSC1-L had taller autumn regrowth than GSC2-H.

A wide range of broad sense  $H^2$  (0.00 to 0.89) was estimated for forage yield across all harvests (Table 2).  $H^2$  for total annual forage dry matter yield over years was 0.93 at Ithaca and 0.57 at Tulelake. Overall  $H^2$  across all harvests and locations was 0.53.

## 4 | DISCUSSION

Dry matter yield is the most important trait for profitable alfalfa production, yet somewhat inexplicably, over the past 30 years, there has been no improvement in on-farm alfalfa yields in the USA (Brummer and Casler, 2014; USDA-NASS, 2022). Genomic selection has been shown to increase the rate of genetic gain in many of the major crops grown in the United States, including alternatives to alfalfa, such as maize (*Zea mays* L.), in livestock rations (Heffner et al., 2010; Beyene et al., 2015; Barre et al., 2022). In this experiment, we evaluated and compared populations developed through traditional recurrent phenotypic selection and genomic selection to investigate whether genomic selection could be a viable option to address the lack of yield improvement in alfalfa.

In this experiment heritability estimates for DMY are higher than have previously been reported (Acharya et al., 2020; Luna-Guerrero et al., 2020), probably due to the use of ten replications within each location of the trial to obtain reliable estimates of forage yield in a densely sown sward.  $H^2$  estimates for Tulelake were lower than Ithaca, due in part to the inclusion of establishment year harvests (which were not measured in Ithaca).

Considering only the populations developed using the genomic prediction model, the GSC1-H population had higher yield than the GSC1-L population, with the population whose parents were chosen randomly (GSC1-R) falling intermediate between the others. Thus, the model had the ability



to shift populations in the expected directions for biomass yield. Across all entries, the GSC1-H population was among the top yielding populations, and GSC1-L was among the lowest yielding.

However, the genomic prediction model appeared to break down on the second cycle of selection, with both the high and low GSC2 populations performing poorly. This is concerning, as one of the major benefits of genomic selection is the potential ability to conduct multiple cycles of GS in the span of a phenotypic selection cycle. However, if the model breaks down after a single cycle, this benefit cannot be realized. Nevertheless, conducting a single cycle of GS in the space of a year is still considerably faster than a PS cycle. Approximately 9000 markers were used for the first cycle of genomic selection and fewer were used for the second cycle, which may explain some of the poor performance of the C2 populations; alternatively, the relative value of marker loci could have shifted following the first cycle, so that the model is simply not useful. In addition, there may have been an inadvertent shift in the dormancy of the C2 populations, which could contribute to lower total DMY. The base population and all populations developed through genomic selection were included in a separate trial investigating the autumn height of various alfalfa populations, a proxy for autumn dormancy. The GSC2-H population was significantly shorter than the GSC1-L population (Figure 2) indicating selection for plants containing alleles for less fall growth. Future applications of genomic selection should include selection criteria to ensure fall dormancy remains unchanged (or that undesirable changes do not occur) during the selection process. This also shows a potential risk of genomic selection in a breeding program – the possibility for undesired shifts of non-target traits. Breeders should be aware of this when making selection decisions, and this result highlights the need to measure other traits of importance during the breeding process.

The GS model was developed based solely on phenotypic information from plants grown in Ithaca, and not surprisingly, the selected populations performed better relative to other entries in Ithaca than in Tulelake. This suggests that any potential gains derived from genomic selection

require the inclusion of phenotypic information from the target environment. This observation has potentially significant implications for the viability of incorporating genomic selection into alfalfa breeding. Already suffering from a paucity of breeders and breeding resources, expanding breeding trials to include more environments may not be possible for many breeding programs. Further investigation is required to determine the requisite number of environments that need to be evaluated in order for GS to work robustly.

The lack of separation between the base population and populations selected for high yield, either by PS or GS, in the overall analysis provides some insight into what breeders have experienced over the past 30 years of alfalfa improvement. The selected populations have not increased yield in an experiment designed to replicate a commercial production environment. Notably, however, in Ithaca the GSC1-H population performed better than the PSC1 population (NY1221) that was selected through phenotypic evaluation, even though both relied on yield information from Ithaca in making selections or in developing the GS model.

Regardless, the lack of DMY gain from the base population using either phenotypic or genomic selection remains a significant concern. Further improvements to the predictive model are possible and may yet result in real gains at the commercial production level. The GS populations evaluated in this experiment derived from a predictive model developed using clonally replicated space-plant yield. A poor correlation between individual space-plant yield and DMY of a densely planted sward is often obtained (Annicchiarico et al., 2006), so an alternative approach evaluating the DMY of families in densely planted small plots might be a better approach (Annicchiarico et al., 2015; Andrade et al. 2020). These families can be bulk genotyped (i.e., pooling multiple individuals within each family) to obtain allele frequency marker data rather than individual genotyping calls (Andrade et al., 2020). This method better captures commercial yield in the model so more accurate

predictions can be made, aligns well with current the current breeding methods in alfalfa, and can be implemented alongside family-based recurrent selection.

Genomic selection is still in its infancy in alfalfa; however, our data indicate there is the potential for greater genetic gain with GS than has been obtained with the use of phenotypic selection alone. Significantly more research is required to investigate alternative models and selection strategies across the wide range of environments in which alfalfa is grown. With the cost of genotyping decreasing, new high throughput technologies being developed, and a greater understanding of the alfalfa genome, the potential for GS to improve yield is quite high. The results of this work will be beneficial not only to alfalfa production but also will help guide decision making for breeding of other outcrossing perennial forages.

## 5 | ACKNOWLEDGEMENTS

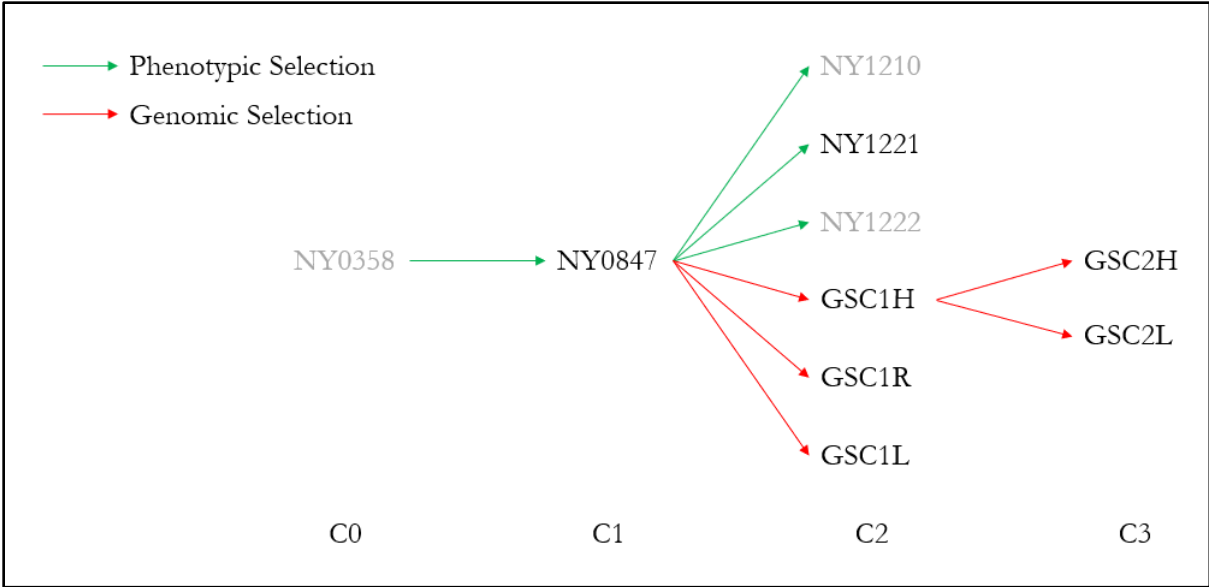
We thank the US Alfalfa Farmer Research Initiative and the California Crop Improvement Association for support of this research to ECB and PGG Wrightson Seeds for a fellowship supporting MF. We thank Darrin Culp and Rob Wilson at the UC ANR Intermountain Research and Extension Center, Tulelake, CA for assistance with plot maintenance, harvest, and data collection. We thank Dusty Pittman for assistance with greenhouse seed production of GS populations.

- Acharya, J. P., Lopez, Y., Gouveia, B. T., de Bem Oliveira, I., Resende, M. F., Muñoz, P. R., & Rios, E. F. (2020). Breeding Alfalfa (*Medicago sativa* L.) Adapted to Subtropical Agroecosystems. *Agronomy*, *10*, 742. doi:<https://doi.org/10.3390/agronomy10050742>
- Andrade, M. H., Acharya, J. P., Benevenuto, J., de Bem Oliveira, I., Lopez, Y., Munoz, P., . . . Rios, E. F. (2022). Genomic prediction for canopy height and dry matter yield in alfalfa using family bulks. *The Plant Genome*, *15*. doi:<https://doi.org/10.1002/tpg2.20235>
- Annicchiarico, P. (2006). Prediction of indirect selection for seed and forage yield of lucerne based on evaluation under spaced planting. *Plant Breeding*, *125*, 641-643. doi:<https://doi.org/10.1111/j.1439-0523.2006.01295.x>
- Annicchiarico, P., & Pecetti, L. (2021). Comparison among nine alfalfa breeding schemes based on actual biomass yield gains. *Crop Science*, *61*, 2355-2370. doi:<https://doi.org/10.1002/csc2.20464>
- Annicchiarico, P., Barrett, B., Brummer, E. C., Julier, B., & Marshall, A. H. (2015). Achievements and challenges in improving temperate forage legumes. *Critical Reviews in Plant Sciences*, *34*, 327-380. doi:<https://doi.org/10.1080/07352689.2014.898462>
- Barre, P., Asp, T., Byrne, S., Casler, M., Faville, M., Rognli, O. A., . . . Ghesquière, M. (2022). Genomic prediction of complex traits in forage plant species: perennial grasses case. *Methods in Molecular Biology*, *2467*, 521-541. doi:10.1007/978-1-0716-2205-6\_19. PMID: 35451789.
- Beyene, Y., Semagn, K., Mugo, S., Tarekegne, A., Babu, R., Meisel, B., . . . Crossa, J. (2015). Genetic gains in grain yield through genomic selection in eight bi-parental maize populations under drought stress. *Crop Science*, *55*, 154-163. doi:<https://doi.org/10.2135/cropsci2014.07.0460>

- Biazzi, E., Nazzicari, N., Pecetti, L., Brummer, E. C., Palmonari, A., Tava, A., & Annicchiarico, P. (2017). Genome-wide association mapping and genomic selection for alfalfa (*Medicago sativa*) forage quality traits and *M. truncatula* species. *PLoS ONE*, *12*(1).  
doi:<https://doi.org/10.1371/journal.pone.0169234>
- Brummer, E. C., & Casler, M. D. (2014). Cool-season forages. In S. Smith, B. Diers, J. Specht, & B. Carver (Eds.), *Yield Gains in Major U.S. Field Crops* (pp. 33-52). Madison, WI: ASA, CSSA, SSSA.
- Butler, D. (2023). asreml: Fits the Linear Mixed Model. doi:[www.vsni.co.uk](http://www.vsni.co.uk)
- Gezan, S. A. (2023). The great debate: Fixed vs. random blocks in experimental design  
<https://vsni.co.uk/blogs/are-blocks-fixed-or-random>.
- Heffner, E. L., Lorenz, A. J., Jannink, J., & Sorrells, M. E. (2010). Plant breeding with genomic selection: genetic gain per unit time and cost. *Crop Science*, *50*, 1681-1690.  
doi:<https://doi.org/10.2135/cropsci2009.11.0662>
- Hothorn, T., Bretz, F., & Westfall, P. (2008). Simultaneous inference in general parametric models. *Biometric Journal*, *50*(3), 346-363.
- Hoyos-Villegas, V., O'Connor, J. R., Heslop, A. D., Hilditch, A., Jahufer, Z. Z., & Barrett, B. A. (2019). Rate of genetic gain for persistence to grazing and dry matter yield in white clover across 90 years of cultivar development. *Crop Science*, *59*, 537-552.  
doi:<https://doi.org/10.2135/cropsci2018.07.0471>
- Lamb, J. F., Sheaffer, C. C., Rhodes, L. H., Sulc, R. M., Undersander, D. J., & Brummer, E. C. (2006). Five decades of alfalfa cultivar improvement: impact on forage yield, persistence, and nutritive value. *Crop Science*, *46*, 902-909. doi:<https://doi.org/10.2135/cropsci2005.08-0236>
- Lenth, R. V. (2021). emmeans: Estimated Marginal Means, aka Least-Squares Means.  
doi:<https://CRAN.R-project.org/package=emmeans>

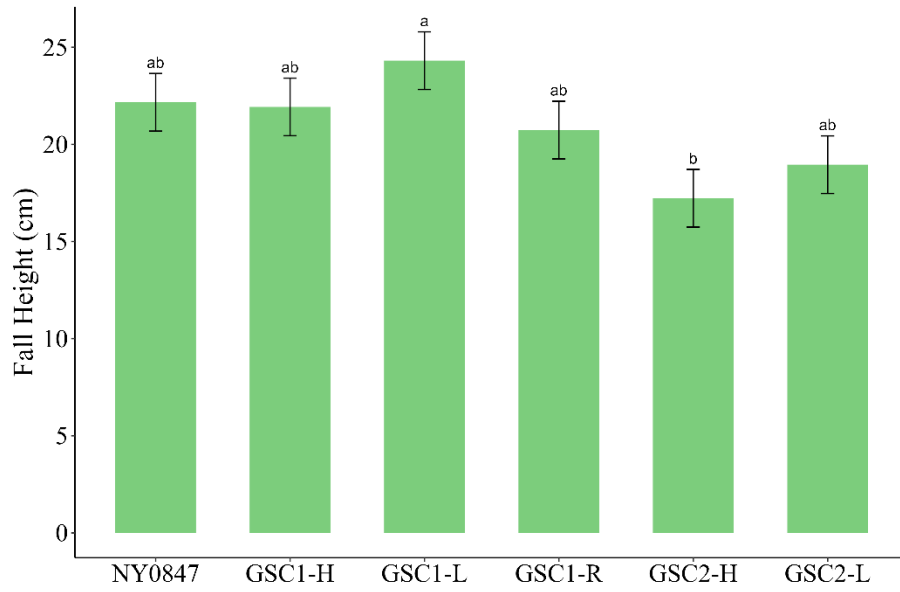
- Li, X., & Brummer, E. C. (2009). Inbreeding depression for fertility and biomass in advanced generations of inter- and intrasubspecific hybrids of tetraploid alfalfa. *Crop Science*, *49*, 13-19. doi:<https://doi.org/10.2135/cropsci2008.04.0205>
- Li, X., & Brummer, E. C. (2012). Applied genetics and genomics of alfalfa breeding. *Agronomy*, *2*, 40-61. doi:<https://doi.org/10.3390/agronomy2010040>
- Li, X., Wei, Y., Acharya, A., Hansen, J. L., Crawford, J. L., Viands, D. R., . . . Brummer, E. C. (2015). Genomic prediction of biomass yield in two selection cycles of a tetraploid alfalfa breeding population. *The Plant Genome*, *8*. doi:<https://doi.org/10.3835/plantgenome2014.12.0090>
- Luna-Guerrero, M. J., López-Castañeda, C., & Hernández-Garay, A. (2020). Genetic improvement of aerial alfalfa biomass and its components: half-sib family selection. *Rev Mex Cienc Pecun*, *11*(4), 1126-1141. doi:<https://doi.org/10.22319/rmcp.v11i4.5344>
- Martin, N. P., & Mertens, D. R. (2005). Reinventing alfalfa for dairy cattle and novel uses. *California Alfalfa and Forage Symposium*. Visalia, CA: UC Cooperative Extension, Agronomy Research and Extension Center.
- McDonagh, J., O'Donovan, M., McEvoy, M., & Gilliland, T. J. (2016). Genetic gain in perennial ryegrass (*Lolium perenne*) varieties 1973 to 2013. *Euphytica*, *212*, 187-199. doi:<https://doi.org/10.1007/s10681-016-1754-7>
- Medina, C. A., Hawkins, C., Liu, X., Peel, M., & Yu, L. (2020). Genome-wide association and prediction of traits related to salt tolerance in autotetraploid alfalfa (*Medicago sativa* L.). *International Journal of Molecular Sciences*, *21*(9), 3361. doi:<https://doi.org/10.3390/ijms21093361>
- Medina, C. A., Kaur, H., Ray, I., & Yu, L. (2021). Strategies to increase prediction accuracy in genomic selection of complex traits in alfalfa (*Medicago sativa* L.). *Cells*, *10*(12), 3372. doi:<https://doi.org/10.3390/cells10123372>

- Meyer, R. D., Marcum, D. B., Orloff, S. B., & Schmierer, J. L. (2007). Alfalfa Fertilisation Strategies. In C. G. Summers, & D. H. Putnam (Eds.), *Irrigated Alfalfa Management for Mediterranean and Desert Zones* (pp. 73-87). Oakland: University of California Agriculture and Natural Resources Publication 8287. doi:<http://alfalfa.ucdavis.edu/IrrigatedAlfalfa>
- Putnam, D. H., Summers, C. G., & Orloff, S. B. (2007). Alfalfa production systems in California. In C. G. Summers, & D. H. Putnam (Eds.), *Irrigated Alfalfa Management for Mediterranean and Desert Zones* (pp. 1-18). Oakland: University of California Agriculture and Natural Resources Publication 8287. doi:<http://alfalfa.ucdavis.edu/IrrigatedAlfalfa>
- RStudio Team. (2020). RStudio: Integrated Development for R. doi:<http://www.rstudio.com/>
- USDA-NASS. 2024. Quick Stats. <https://quickstats.nass.usda.gov/> (accessed 20 Feb 2024).
- Woodfield, D. R., & Caradus, J. R. (1994). Genetic improvement in white clover representing six decades of plant breeding. *Crop Science*, *34*, 1205-1213.  
doi:<https://doi.org/10.2135/cropsci1994.0011183X003400050011x>



**FIGURE 1** Populations developed and evaluated as part of this trial.





**FIGURE 2** Estimated marginal means and standard errors for fall height for each population. Multiple pairwise comparisons adjusted using the Tukey method. Different letters denote significant differences in fall height between populations ( $p < 0.05$ ).

**TABLE 1** Average forage dry matter yield across all harvests (Overall) and for spring, summer, or fall harvests across locations and within each location.

Population	<u>All Locations (Mg DM ha<sup>-1</sup>)</u>				<u>Ithaca, NY (Mg DM ha<sup>-1</sup>)</u>				<u>Tulelake, CA (Mg DM ha<sup>-1</sup>)</u>			
	Overall	Spring	Summer	Fall	Overall	Spring	Summer	Fall	Overall	Spring	Summer	Fall
<b>NY0847</b>	4.96 <sup>a</sup>	6.54 <sup>a</sup>	5.20 <sup>a</sup>	3.60 <sup>a</sup>	4.51 <sup>ab</sup>	5.67 <sup>ab</sup>	4.47 <sup>a</sup>	3.41 <sup>a</sup>	5.42 <sup>a</sup>	7.40 <sup>a</sup>	5.93 <sup>a</sup>	3.79 <sup>ab</sup>
<b>NY1221</b>	4.83 <sup>ab</sup>	6.41 <sup>a</sup>	5.06 <sup>abc</sup>	3.47 <sup>ab</sup>	4.31 <sup>bc</sup>	5.35 <sup>abcd</sup>	4.33 <sup>ab</sup>	3.26 <sup>ab</sup>	5.36 <sup>ab</sup>	7.47 <sup>a</sup>	5.79 <sup>ab</sup>	3.68 <sup>b</sup>
<b>GSC1-H</b>	4.97 <sup>a</sup>	6.63 <sup>a</sup>	5.19 <sup>a</sup>	3.48 <sup>ab</sup>	4.54 <sup>a</sup>	5.82 <sup>a</sup>	4.49 <sup>a</sup>	3.32 <sup>a</sup>	5.40 <sup>a</sup>	7.43 <sup>a</sup>	5.88 <sup>a</sup>	3.63 <sup>b</sup>
<b>GSC1-L</b>	4.67 <sup>cd</sup>	5.95 <sup>c</sup>	4.92 <sup>c</sup>	3.60 <sup>a</sup>	4.16 <sup>c</sup>	4.98 <sup>cd</sup>	4.26 <sup>ab</sup>	3.24 <sup>ab</sup>	5.18 <sup>b</sup>	6.93 <sup>b</sup>	5.58 <sup>b</sup>	3.96 <sup>a</sup>
<b>GSC1-R</b>	4.86 <sup>ab</sup>	6.33 <sup>ab</sup>	5.12 <sup>ab</sup>	3.54 <sup>ab</sup>	4.40 <sup>ab</sup>	5.50 <sup>abc</sup>	4.38 <sup>ab</sup>	3.31 <sup>a</sup>	5.32 <sup>ab</sup>	7.16 <sup>ab</sup>	5.85 <sup>ab</sup>	3.76 <sup>ab</sup>
<b>GSC2-H</b>	4.58 <sup>d</sup>	6.00 <sup>bc</sup>	4.88 <sup>c</sup>	3.26 <sup>c</sup>	3.91 <sup>d</sup>	4.80 <sup>d</sup>	3.97 <sup>c</sup>	2.95 <sup>c</sup>	5.26 <sup>ab</sup>	7.20 <sup>ab</sup>	5.79 <sup>ab</sup>	3.56 <sup>b</sup>
<b>GSC2-L</b>	4.74 <sup>bc</sup>	6.35 <sup>abc</sup>	4.91 <sup>bc</sup>	3.38 <sup>bc</sup>	4.15 <sup>cd</sup>	5.18 <sup>bcd</sup>	4.18 <sup>bc</sup>	3.07 <sup>bc</sup>	5.33 <sup>ab</sup>	7.51 <sup>a</sup>	5.65 <sup>ab</sup>	3.68 <sup>ab</sup>

*Note:* Multiple pairwise comparisons adjusted using the Tukey method. Different letters within columns denote significant differences for forage yield between populations ( $p < 0.05$ ).

**TABLE 2** Broad-sense heritability ( $H^2$ ) for forage yield by harvest.

Harvest	<u>2017</u>		<u>2018</u>		<u>2019</u>		<u>2020</u>	
	CA	NY	CA	NY	CA	NY	CA	NY
1	0.00	-	0.64	0.72	0.69	0.24	0.40	0.84
2	0.49	-	0.80	0.61	0.25	0.71	-	0.85
3	0.00	-	0.23	0.69	0.00	0.72	-	0.89
4	-	-	0.62	-	0.34	-	-	-

Abbreviations: CA = Tulelake, CA; NY = Ithaca, NY.

# Chapter 3

## Genomic Selection for Forage Dry Matter Yield in Alfalfa Using Family Bulks

### 1 | INTRODUCTION

Alfalfa (*Medicago sativa* L.) is one of the most important perennial forage crops in the world. It is the third most valuable field crop in the United States in which California leads the nation for hay and seed production, generating in excess of \$1B in 2022 (United States Department of Agriculture [USDA], 2023; California Department of Food and Agriculture [CDFA], 2023). Its high yield and nutritional value are key drivers for California's dairy industry, the state's top valued agricultural commodity (CDFA, 2023). In addition to its economic value and importance as a forage, alfalfa provides a host of beneficial ecosystem services. Its nitrogen fixing capabilities and perennial nature promote sustainable cropping systems and contribute to nutrient cycling (Andrade et al., 2022). Alfalfa also plays a role as an important insectary and habitat for native fauna (Putnam et al., 2007). Cultivated alfalfa is predominantly derived from the subsp. *sativa*, an allogamous autotetraploid ( $2n = 4x = 32$ ). It is commercialized as synthetic populations consisting of highly variable and heterozygous plants (Annicchiarico & Pecetti, 2021).

Alfalfa breeding programs are based on recurrent phenotypic selection, with or without progeny testing (Li & Brummer, 2012). They are designed to increase the frequency of desirable alleles in a population while maintaining genetic variability for continued genetic improvement (Hallauer et al., 2010). Breeding goals in alfalfa are characteristic of those in other crops: increasing yield, enhancing forage quality, and improving tolerance to biotic and abiotic stresses (Li &

Brummer, 2012). Simply inherited traits with high heritability have been greatly improved through traditional breeding methods; however, improvement in complex, quantitatively inherited traits have been less successful (Li et al., 2012), most notably yield for which there has been little to no improvement over the last 30 years (Brummer & Casler, 2014). Long breeding cycles (3-5 years), multiple harvests per year, limited breeding resources, inability to make gains in the harvest index, significant genotype by environment interaction ( $G \times E$ ), and selection based on vigor of spaced plants or short family rows (versus measurements of yield on plots grown at stand densities used in commercial production) are all factors contributing to the low rate of yield progress (Li & Brummer, 2012; Acharya et al., 2020). Yield improvements in alfalfa in the past can be mainly attributed to improvement of ‘defensive’ traits i.e., improvements in pest and disease resistance (Lamb et al., 2006). This helps alfalfa reach its yield potential, but it does not result in an increase in yield *per se*. To select on yield *per se*, selections could be based on yield data from the first full year of production before plant mortality becomes an influencing factor impacting yield, while persistence could be evaluated at the end of a multi-year trial.

Marker-assisted selection (MAS) is a modern tool that has great potential in addressing the lack of genetic gain in alfalfa yield (Brummer, 2004). The availability of a large number of single nucleotide polymorphism (SNP) markers, cost effective genotyping assays, and the recent availability of chromosome-scale, haplotype-phased genome assemblies (Chen et al., 2020; Shen et al., 2020) facilitate the dissection of complex traits and provide a pathway for genetic improvement (Li et al., 2012). Genomic selection (GS), first introduced by Meuwissen et al. (2001), has been adopted by alfalfa breeding programs in recent years for improvement of a variety of complex traits (Li et al., 2015; Biazzi et al., 2017; Jia et al., 2018). By enabling breeders to perform marker-only selection, a cycle of selection could be completed in less than a year, addressing one of the biggest impediments to faster genetic gain – the need for multi-year evaluations (Li & Brummer, 2012). The majority of

GS applications in alfalfa to date are based on phenotypic and genotypic data at the individual plant level (Andrade et al., 2022). However, alfalfa is marketed as synthetic cultivars with breeding based on the evaluation of families, not individual plants. This is particularly relevant when attempting to evaluate yield. An alternative approach to GS is pooling individuals and genotyping family bulks rather than individuals (Cericola et al., 2018; Annicchiarico & Pecetti, 2021; Andrade et al., 2022). Phenotypic data can then be collected at the family level from densely planted plots, a better representation of commercial yield, and the relationship matrix is built based on allele frequency marker data, rather than individual genotyping calls.

High quality phenotypic data is essential for maximizing the predictive ability of a GS model (Voss-Fels et al., 2018). Field trials are inherently variable, particularly in the case of perennial crops with multiple harvests where spatial and temporal differences can have a large impact the quality of phenotypic data. Randomization, blocking, and complex trial designs are commonly used to account for field variation; however, they are often inadequate to account for all the spatial trends in large experiments (Gilmour et al., 1997). Residual maximum likelihood (REML) has become commonplace in plant breeding to estimate variance components and calculate genetic parameters using linear mixed models (Biswas et al., 2021). Best linear unbiased predictions (BLUPs) of random effects have proven effective in predicting breeding values to guide breeding decisions (Bernardo, 2020). Mixed-model analysis has the advantage of handling unbalanced data and can incorporate additional information using covariance matrices, providing more accurate predictions (Bernardo, 2020). In field trials, experimental units in close proximity to one-another are expected to be more highly correlated than those far apart, and successive harvests likely will be more highly correlated than harvests separated by a greater time interval. Therefore, a better way of managing spatial and temporal variation is to include covariance matrices in the analysis to account for spatial and temporal trends in the data (Faveri et al., 2015).

The long-term goal of this research was to address the lack of yield improvement in alfalfa through the incorporation of modern breeding methodology. The specific objectives of this study were to develop and assess the performance of a GS model for biomass yield developed through the genotyping of family bulks and improved phenotyping methods to capture spatial and temporal variation in alfalfa breeding trials.

## 2 | MATERIALS AND METHODS

### 2.1 | Training populations

The training populations used in this study were part of the UC Davis elite non-dormant alfalfa breeding program. Plants were selected from an evaluation trial in Davis, CA and crossed in the greenhouse in 2018 resulting in two populations – UCAL1960 and UCAL1970. In 2019, seed from both populations was germinated in the greenhouse and transplanted to the field at the UC Davis Plant Sciences Farm. Approximately 200 plants from each population were grown in adjacent areas that were each placed under mesh cages to enable pollination with leaf cutter bees (*Megachile rotundata*). The seed from each plant was harvested individually and the highest seed yielding families of each population were included in the trial. The entries consisted of 105 half-sib families from UCAL1960, 88 half-sib families from UCAL1970, balanced bulks from each population, and three cultivar checks: Highline, UC Impalo and CUF 101.

### 2.2 | Field trial

A field trial was established at two locations, one in Yolo County (38°32'08.8"N 121°46'31.5"W) and the other in Solano County (38°31'39.2"N 121°46'18.1"W), on the UC Davis Plant Sciences Farm near Davis, CA in May 2020. Although the two sites are close together, the Solano location is in a

floodplain of the nearby Putah Creek. The locations are in a Mediterranean environment characterized by hot, dry summers, cool winters, and moderate annual rainfall, which falls predominantly in the cooler months from November to March (National Oceanic and Atmospheric Administration [NOAA], 2023). The soil type is a Yolo silt clay loam (fine-silty, mixed, superactive, thermic Fluventic Haploxerepts). Soil tests were conducted prior to planting to adjust P, K, and pH according to soil test recommendations. Seeds were sown in trays in the greenhouse in March 2020 and seedlings transplanted in early May. The trial at each location was a randomized complete block design consisting of two replicates, each with 7 rows and 29 ranges. Plots consisted of 24 plants laid out in a regular  $4 \times 6$  grid with 20 cm spaces between plants. There was a 30 cm space between rows and a 110 cm space between ranges to allow room for mechanical harvesting. This trial was managed as a high-yielding alfalfa stand; soil tests were conducted each year, with amendments made accordingly. The trial was initially irrigated using sprinklers, but after plants were well established, flood irrigation was used to match crop evapotranspiration. Weeds were managed by a combination of manual removal and herbicides, and insect pests were monitored and controlled with insecticide as needed.

### 2.3 | Phenotyping

Dry matter yield (DMY) was measured by mechanical harvest when the plants had reached 10% bloom. A self-propelled forage plot harvester with a flail chopper and weigh bin was used to cut plots uniformly at 7.5 cm and measure the fresh weight. There was a total of 12 harvests, three in the establishment year, seven in the first full year of production (2021), and two in the second production year (2022). Each harvest, 20 hand grab samples (~500 g wet weight) were collected throughout the day of harvest and weighed before being dried at 60°C in a forced-air drying oven for 7 days. Dry samples were then weighed and used to calculate an average dry matter percentage.



Plot yield was recorded on a dry matter basis ( $\text{kg DM ha}^{-1}$ ) after adjusting the fresh weights with the average dry matter percentage.

## 2.4 | Genotyping

The first fully expanded trifoliolate leaf from the growing tip of the plant was collected from every plant within each family (i.e., from one plot) from a single replication of the trial and pooled for DNA extraction. Subsequently, tissue samples were lyophilized and ground. DNA was extracted from the ground tissue using DNeasy 96 plant kits quantified using a BioTek Synergy H1 Microplate Reader with Take3 Micro-volume plate. Genotyping-by-sequencing (GBS) libraries were constructed using the protocol of Elshire et al. (2011) modified as described previously in Li et al. (2014). Briefly, 100 ng of DNA from each sample was digested with PstI enzyme. Two different types of adapters, a barcode adapter and common adapter, were ligated with the DNA fragment using T4 DNA ligase. Equal volumes of the ligation product from each sample were pooled together and purified using AMPure XP beads (Beckman Coulter). The ligation product was then amplified with the Phusion High-Fidelity PCR Master mix (Thermo Scientific), using 50 ng of DNA as the template and 25 nmole of each PCR primer in a 50  $\mu\text{l}$  reaction system. PCR was performed under the following thermal cycling conditions: 72°C for 5 min, denaturation at 98°C for 30 sec, followed by 12 cycles with 10 sec of denaturation at 98°C, 30 sec of primer annealing at 65°C and 30 sec extension at 72°C. The resulting library was again purified using the AMPure XP beads (Beckman Coulter). The DNA fragment sizes were checked using a Bioanalyzer (Agilent) then all families were pooled and sequenced with Illumina Nextseq.

The raw reads were compressed using clumpify software from the BBTools package (Bushnell, 2015), then trimmed using Cutadapt (Martin, 2011). The Tassel 5.0 GBS v2 pipeline was used for SNP discovery (Bradbury et al., 2007). Default parameters were used for all steps unless

otherwise stated. First, tags and taxa are identified from the raw FastQ files and stored in a local database using GBSSeqToTagDBPlugin. Next, distinct tags from the database were retrieved and reformatted to be aligned with the reference genome using the TagExportToFastqPlugin. Bowtie2 v2.5.1 (Langmead & Salzberg, 2012)) was used to align the filtered reads with the *Medicago sativa* reference genome developed by Shen et al. (2020) using the --very-sensitive-local input. The resulting SAM file created from this step was then used to update the local database with the SAMToGBSdbPlugin. DiscoverySNPCallerPluginV2 then identified SNPs from the aligned tags and updated the database. Finally, ProductionSNPCallerPluginV2 was used to generate a VCF formatted genotype file. SNPs were further filtered with VCFTools (Danecek et al., 2011) using the following criteria: biallelic variants only, minor allele frequency > 0.05, minimum sequencing depth for each data point > 4, minimum mean read depth over all samples > 30, and maximum missing data of 10%. After filtering, 39,229 SNPs remained for downstream analysis and genomic prediction.

Read counts for reference and alternative alleles were extracted from the VCF file and used to calculate an allele frequency matrix. Allele frequency was calculated by:

$$A/(A + a)$$

where  $A$  is the allele count of the alternative allele, and  $a$  is the allele count of the reference allele.

## 2.5 | Phenotypic data analysis

All analyses were performed using R statistical software and ASReml-R v4.0 in RStudio v4.1.0 (Butler et al., 2017; R Development Core Team, 2018; RStudio Team, 2020). Best linear unbiased predictions (BLUPs) were calculated for each trial entry following the process for spatial and temporal analysis of multi-harvest data by De Faveri et al. (2015). Analyses were first broken down

into individual harvests at each location, followed by a multi-harvest model. The individual harvest model was fit as follows:

$$y = \mu + X + Z_1 r + Z_2 c + Z_3 f + \varepsilon,$$

where,  $y$  is the vector of phenotypic values for DMY;  $\mu$  is the overall mean;  $X$  and  $Z$  represent the incidence matrices for fixed and random effects respectively;  $r$  is the random effect vector of rows, with  $r \sim N(0, I\sigma_r^2)$  and  $\sigma_r^2$  is the variance of row within location;  $c$  is the random effect vector of columns, with  $c \sim N(0, I\sigma_c^2)$  and  $\sigma_c^2$  is the variance of column within location;  $f$  is the random effect vector of family within location, with  $f \sim N(0, I\sigma_f^2)$  and  $\sigma_f^2$  is the variance of family;  $I$  is the identity matrix;  $\varepsilon$  is the random vector of residuals,  $\varepsilon \sim N(0, R\sigma_\varepsilon^2)$ , in which  $R$  is the covariance matrix of  $\varepsilon$ , and it is defined as:  $R = \sigma^2 \Sigma_c \otimes \Sigma_r$ , where  $\Sigma_c$  and  $\Sigma_r$  are the spatial correlation matrices within location for column and row that were assumed to arise from an autoregressive process of order 1, and  $\otimes$  denotes the Kronecker product. This residual structure allows correlation in both the row and column direction, thereby accounting for the spatial trend in which data from plots close to one another are more similar than those further apart.

The multi-harvest model was fitted using harvest as repeated measures producing a single genotypic value across all harvests for the first full year of production. The following model was used:

$$y = \mu + X_1 h + Z_1 r + Z_2 c + Z_3 f h + \varepsilon,$$

Where  $y$  is the vector of phenotypic values for DMY,  $\mu$  is the overall mean,  $X$  and  $Z$  represent the incidence matrices for fixed and random effects respectively,  $h$  is the fixed effect of harvest,  $r$  is the random effect vector of rows, with  $r \sim N(0, I\sigma_r^2)$  and  $\sigma_r^2$  is the variance of row;  $c$  is the random effect vector of columns, with  $c \sim N(0, I\sigma_c^2)$  and  $\sigma_c^2$  is the variance of column within location;  $f h$

is the random effect vector of family within each harvest, with  $fh \sim N(0, G \otimes I)$ ;  $I$  is the identity matrix;  $G$  is the genetic variance/covariance matrix and was modeled using a second order ante-dependence model that allows for serial correlation within entries over repeated measurements;  $\varepsilon$  is the random vector of residuals,  $\varepsilon \sim N(0, R\sigma_e^2)$ , in which  $R$  is the residual variance/covariance matrix and it is defined as:  $R = \sigma^2 \Sigma_c \otimes \Sigma_r$ , where  $\Sigma_c$  and  $\Sigma_r$  are the spatial correlation matrices within location for column and row that were assumed to arise from an autoregressive process of order 1, and  $\otimes$  denotes the Kronecker product.

## 2.5 | Linkage disequilibrium and population structure analysis

Pairwise Pearson correlation between pairs of SNPs were computed to assess the linkage disequilibrium within and across chromosomes using Tassel 5.0 (Bradbury et al., 2007). The LD decay over physical distance was determined as the mean distance under a threshold of  $r^2 = 0.2$ .

Population structure was determined by performing a principal component analysis (PCA) of the allele frequency-based relationship matrix of all 198 trial entries using R (R Development Core Team, 2018).

## 2.5 | Genomic prediction model development

The allele frequency genomic relationship matrix was used to calculate genomic estimated breeding values (GEBVs) for each of the trial entries following the ‘method 1’ from VanRaden (2008). The relationship matrix is comprised of frequency estimates from the GBS data with SNPs in rows and samples in columns,  $X_{ij}$  being the allele frequency at SNP  $i$  in family  $j$ , and  $M$  is the matrix centered by SNP mean frequencies,  $M = M_{ij} = X_{ij} - \bar{X}_i$ . Missing SNP data was substituted by the mean SNP frequency i.e., a mean imputation for missing genotypes. The G-matrix is:

$$G = \frac{M'M}{K},$$

where  $K$  is the sum of expected SNP variances  $\Sigma \bar{X}_i(1 - \bar{X}_i)$ . The resulting G-matrix was singular which means that it is non-invertible. To solve this issue, we computed the eigen-decomposition  $G = E'_v \Lambda^{-1} E_v$  and changed the last close to zero eigenvalue to a value just below the second-to-last eigenvalue. We then computed the inverse of  $G$  by:

$$G_{inv} = E'_v \Lambda^{-1} E_v.$$

ASReml-R was then used to calculate genomic best linear unbiased predictions with the first two principal components included to account for population structure (Butler et al., 2017).

## 2.6 | Cross-validation and predictive ability

The cross-validation scheme to assess the predictive ability of the GS model randomly assigned 90% of the families to the training set and 10% as the validation set. The predictive ability of the model was obtained by calculating the Pearson correlation between predicted and adjusted phenotypes. This process was then repeated 10 times using different training sets. Each location was assessed independently as well as an overall analysis across harvests and locations.

## 3 | RESULTS

### 3.1 | Phenotypic data

The mean forage DMY across all 12 harvests ranged from 2592 kg DM ha<sup>-1</sup> in the tenth harvest in September 2021 to 6381 kg DM ha<sup>-1</sup> in the 5<sup>th</sup> harvest in April 2021 (Fig. 1). The data follow a trend, with higher yielding harvests occurring in the earlier spring harvests and then declining through summer into early autumn.

The phenotypic correlation among harvests for DMY ranged from 0.06 to 0.86, with successive harvests having higher correlations than harvests further apart, as would be expected (Fig. 2). The temporal correlation between harvests was accounted for in the model by the inclusion of the second order ante-dependance structure on the genetic variance/covariance matrix.

Heritability for biomass yield in the Solano County location was consistent with previous studies at around 0.29 (Andrade et al., 2022). Significant spatial variation in the yolo county location resulted in large standard errors and a low heritability of 0.04.

### 3.2 | Molecular data and population structure

Linkage disequilibrium decayed at distances of 130 kbp based on the threshold of  $r^2 = 0.2$  (Fig. 3), which is congruent with similar studies in outcrossing perennial forages (Andrade et al., 2020; Inostroza et al., 2018).

The allele-frequency based genomic relationship matrix was used to perform a principal component analysis. The first two principal components explained 73% of the variation (Fig. 4). These results show strong population structure, which is somewhat unexpected as the populations are both closely related and originate from older UC Davis breeding germplasm, including the three check cultivars. UCAL 1960 underwent several cycles of selection in Davis and El Centro for resistance to various diseases and pests, which may be the cause of its differentiation.

### 3.3 | Genomic selection

Only the seven harvests in the first full year of production from Solano County were chosen to build the final genomic prediction model. The significant spatial variation and low heritability from the Yolo County location resulted in lower prediction accuracies when included. Forage yield in the establishment year typically does not represent production in subsequent years, so the first three

harvests (those from 2020) were excluded. Further, as stands age, plant mortality due to diseases and other biotic sources and due to abiotic causes occurs, such that biomass yield measurements do not reflect yield potential as much as resistance or tolerance to these stressors. In an attempt to assess true yield performance rather than resistance to biotic and abiotic, we only used the seven harvests in 2021 for the model (and in fact, we did not even measure yield after the second harvest in 2022 due to the mortality we observed). Predictive ability varied across the range of individual harvests with harvest three in May 2021 having the highest predictive ability of 0.27 and harvest four in June 2021 having the lowest at 0.04. The combination of all harvests in 2021 resulted in a predictive ability of 0.30, consistent with Andrade et al. (2022), who utilized similar methods of family bulks for non-dormant alfalfa populations in the south-eastern united states.

#### 4 | DISCUSSION

Alfalfa has suffered from a lack of yield improvement over the last 30 years of breeding and research. Traditional recurrent phenotypic selection, although useful for the improvement of simply inherited traits, has been unsuccessful in translating yield gains to commercial producers. In this study, genomic selection was incorporated into the UC Davis alfalfa breeding program across two elite non-dormant populations in an effort to select families for improved yield potential. Whereas previous predictive models in alfalfa have been developed based on genotyping single plants and phenotypic information from space plants or short family rows, this study used bulk genotyping of families, as demonstrated by Andrade et al. (2022), and uses phenotypic data on densely transplanted mini-sward plots. The goal was to better capture yield that could be expected in a commercial planting, while reducing genotyping costs, and aligning the genotyping with current breeding

methodology in alfalfa, which is also performed on a family level. To further improve the quality of phenotypic data, spatial and temporal variation were modeled using variance/covariance structures. The predictive ability of GS models is affected by a range of factors, including the number, density and distribution of genetic markers, population size and structure, the degree of LD, and the quality of phenotypic data. The LD decay in this study was moderate (130 kbp), but our marker density (39,229 SNPs distributed across the whole genome) was sufficient to provide coverage of the genome. In a study optimizing whole-genome sequencing in autotetraploid blueberry (Oliveira et al., 2020), the predictive ability of genomic selection increased rapidly with the inclusion of more markers; however, a plateau was reached with 10,000 markers or more. GBS therefore should provide sufficient markers for accurate GS in alfalfa, as we noted previously (Li et al., 2015).

Population structure can induce bias in genomic predictions if not incorporated into the prediction model. Organized breeding programs can develop significant population structure, as observed in this study, where the two populations included in the training population were related, yet clearly distinct. Here we accounted for population structure by including the first two principal components from a PCA in the model.

Population size is perhaps the most important factor influencing the predictive ability of GS. A significant increase in predictive ability for fruit yield in blueberry occurred by increasing population size from 120 to ~1500, with an ideal size of 1000 or more (Oliveira et al. (2020)). We evaluated relatively few families in this study (193) which may have impacted the predictive ability of our model, even though it represented a maximum number of families we could reasonably harvest and manage. High throughput phenotyping could be a solution to greatly increase the number of families evaluated for DMY in alfalfa without a significant increase in cost to the breeder (see Francis, Chapter 4 in this thesis). Remotely assessing biomass from drone-based cameras and



harvesting a subset of plots would allow a breeder to evaluate significantly more families with a similar cost to current phenotyping methods.

High quality phenotypic data is essential for producing accurate genomic predictions. The correlation between harvests was significant and positive in this study, and spatial variation is inherent in field trials. Accounting for spatial variation and temporal correlation between repeated measures improves the quality of phenotypic data, thus allowing for more accurate predictions which may translate to genetic gain.

Moving forward, we intend to generate a range of populations based on both phenotypic and genomic selection from this yield evaluation trial. These will then be evaluated alongside other elite material for DMY to assess if there has been any improvement. Due to the lengthy breeding process of perennial forages, it will take several years to determine whether these methods have been successful. We will continue to develop the predictive model as more material is evaluated and adjust accordingly. To improve the predictive ability of the model moving forward a combination of evaluating a greater number of families and improving the quality of phenotypic data through better modeling will be imposed. Increasing the size of the training population could be facilitated without a significant increase in costs by using modern high-throughput phenotyping tools, such as drone-based remote sensing.

With decreasing costs of genotyping, improved computational software and the availability of genomic resources (such as reference genomes for tetraploid alfalfa), genomic selection is becoming increasingly available to more resource limited breeding programs like alfalfa. There is still much research required to assess whether actual yield gain can be achieved; however, these studies provide a baseline for future studies to investigate potential yield improvement.

## 5 | ACKNOWLEDGEMENTS

We thank the US Alfalfa Farmer Research Initiative and the California Crop Improvement Association for support of this research to ECB and PGG Wrightson Seeds for a fellowship supporting MF.

- Acharya, J. P., Lopez, Y., Gouveia, B. T., de Bem Oliveira, I., Resende, M. F., Muñoz, P. R., & Rios, E. F. (2020). Breeding Alfalfa (*Medicago sativa* L.) Adapted to Subtropical Agroecosystems. *Agronomy*, *10*, 742. doi:<https://doi.org/10.3390/agronomy10050742>
- Andrade, M. H., Acharya, J. P., Benevenuto, J., de Bem Oliveira, I., Lopez, Y., Munoz, P., . . . Rios, E. F. (2022). Genomic prediction for canopy height and dry matter yield in alfalfa using family bulks. *The Plant Genome*, *15*. doi:<https://doi.org/10.1002/tpg2.20235>
- Annicchiarico, P., & Pecetti, L. (2021). Comparison among nine alfalfa breeding schemes based on actual biomass yield gains. *Crop Science*, *61*, 2355-2370. doi:<https://doi.org/10.1002/csc2.20464>
- Bernardo, R. (2020). Reinventing quantitative genetics for plant breeding: something old, something new, something borrowed, something BLUE. *Heredity*, *125*, 375-385. doi:<https://doi.org/10.1038/s41437-020-0312-1>
- Biazzi, E., Nazzicari, N., Pecetti, L., Brummer, E. C., Palmonari, A., Tava, A., & Annicchiarico, P. (2017). Genome-Wide Association Mapping and Genomic Selection for Alfalfa (*Medicago sativa*) Forage Quality Traits. *PLOS ONE*, *12*(1). doi:<https://doi.org/10.1371/journal.pone.0169234>
- Biswas, A., Andrade, M. H., Acharya, J. P., de Souza, C. L., Lopez, Y., de Assis, G., . . . Rios, E. F. (2021). Phenomics-assisted selection for herbage accumulation in alfalfa (*Medicago sativa* L.). *Frontiers in Plant Science*, *12*. doi:<https://doi.org/10.3389/fpls.2021.756768>

- Bradbury, P. J., Zhang, Z., Kroon, D. E., Casstevens, T. M., Ramdoss, Y., & Buckler, E. S. (2007). TASSEL: software for association mapping of complex traits in diverse samples. *Bioinformatics*, 23(19), 2633-2635. doi:<https://doi.org/10.1093/bioinformatics/btm308>
- Brummer, E. C. (2004). Genomics research in alfalfa, *Medicago sativa* L. In R. F. Wilson, H. T. Stalker, & E. C. Brummer (Eds.), *Legume Crop Genomics*. Champaign, IL: AOCS Press.
- Brummer, E. C., & Casler, M. D. (2014). Cool-season forages. In S. Smith, B. Diers, J. Specht, & B. Carver (Eds.), *Yield Gains in Major U.S. Field Crops* (pp. 33-52). Madison, WI: ASA, CSSA, SSSA.
- Bushnell, B. (2015). BBMap. Retrieved from [sourceforge.net/projects/bbmap/](https://sourceforge.net/projects/bbmap/)
- Butler, D. G., Cullis, B. R., Gilmour, A. R., Gogel, B. G., & Thompson, R. (2017). *ASReml-R reference manual. Version 4*. Hemel Hempstead, UK: VSN International.
- California Department of Food and Agriculture (CDFA). (2023). Field Crops. In *California Agricultural Statistics Review 2021-2022*. doi:[https://www.cdfa.ca.gov/Statistics/PDFs/2022\\_Ag\\_Stats\\_Review.pdf](https://www.cdfa.ca.gov/Statistics/PDFs/2022_Ag_Stats_Review.pdf)
- Cericola, F., Lenk, I., Fè, D., Byrne, S., Jensen, C. S., Pederson, M. G., . . . Janss, L. (2018). Optimized use of low depth genotyping-by-sequencing for genomic prediction among multi-parental family pools and single plants in perennial ryegrass (*Lolium perenne* L.). *Frontiers in Plant Science*, 9, 369. doi:10.3389/fpls.2018.00369
- Chen, H., Zeng, Y., Yang, Y., Huang, L., Tang, B., Zhang, H., . . . Qiu, Q. (2020). Allele-aware chromosome-level genome assembly and efficient transgene-free genome editing for the autotetraploid cultivated alfalfa. *Nature Communications*, 11, 2494. doi:<https://doi.org/10.1038/s41467-020-16338-x>

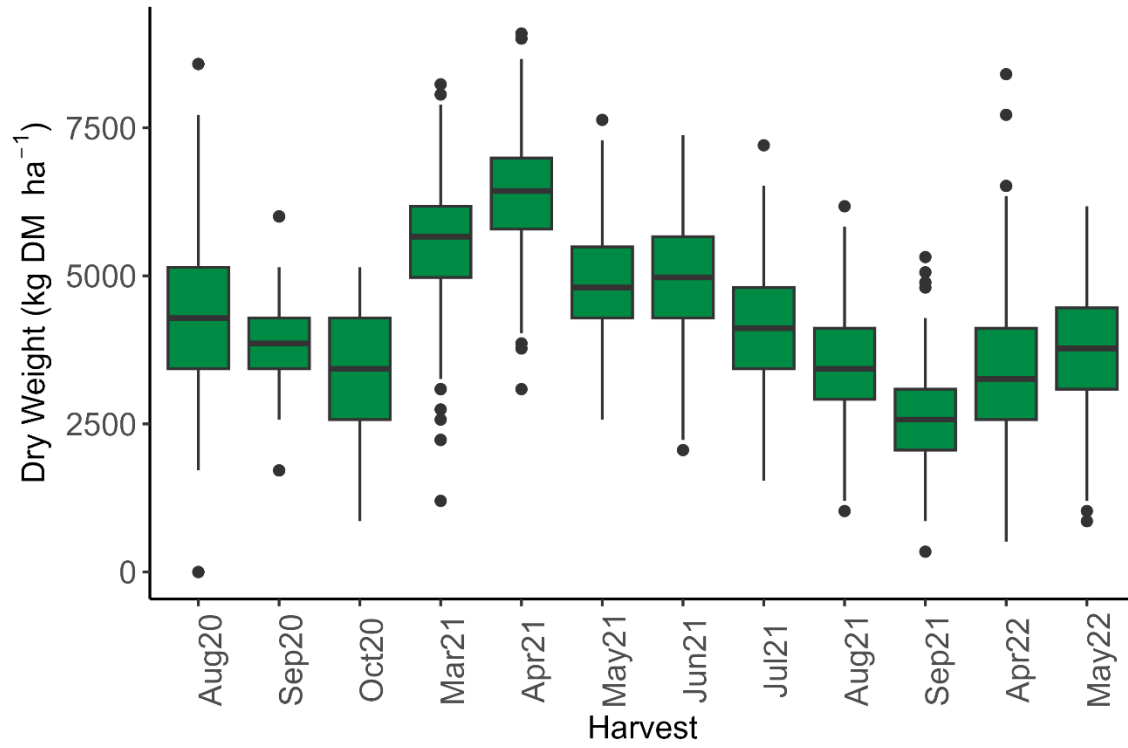
- Danecek, P., Auton, A., Abecasis, G., Albers, C. A., Banks, E., DePristo, M. A., . . . 1000 Genomes Project Analysis Group. (2011). The variant call format and VCFTools. *Bioinformatics*, *27*(15), 2156-2158. doi:<https://doi.org/10.1093/bioinformatics/btr330>
- de Bem Oliveira, I., Amadeu, R. R., Ferrão, L. F., & Muñoz, P. R. (2020). Optimizing whole-genomic prediction for autotetraploid blueberry breeding. *Heredity*, *125*, 437-448. doi:<https://doi.org/10.1038/s41437-020-00357-x>
- de Koning, D. J. (2016). Meuwissen et al. on genomic selection. *Genetics*, *203*(1), 5-7. doi:<https://doi.org/10.1534/genetics.116.189795>
- Elshire, R. J., Glaubitz, J. C., Sun, Q., Poland, J. A., Kawamoto, K., Buckler, E. S., & Mitchell, S. E. (2011). A Robust, Simple Genotyping-by-Sequencing (GBS) Approach for High Diversity Species. *PLOS ONE*, *6*(5). doi:<https://doi.org/10.1371/journal.pone.0019379>
- Faveri, J. D., Verbyla, A. P., Pitchford, W. S., Venkatanagappa, S., & Cullis, B. R. (2015). Statistical methods for analysis of multi-harvest data from perennial pasture variety selection trials. *Crop and Pasture Science*, *66*, 947-962. doi:<https://doi.org/10.1071/CP14312>
- Gilmour, A. R., Cullis, B. R., & Verbyla, A. P. (1997). Accounting for natural and extraneous variation in the analysis of field experiments. *Journal of Agricultural, Biological, and Environmental Statistics*, *2*(3), 269-293. doi:<https://doi.org/10.2307/1400446>
- Hallauer, A. R., Filho, J. B., & Carena, M. J. (2010). Breeding plans. In *Quantitative Genetics in Maize Breeding* (Vol. 6). New York, NY: Springer. doi:[https://doi.org/10.1007/978-1-4419-0766-0\\_12](https://doi.org/10.1007/978-1-4419-0766-0_12)
- Inostroza, L., Bhakta, M., Acuña, H., Vásquez, C., Ibáñez, J., Tapia, G., Mei, W., Kirst, M., Resende Jr, M., & Munoz, P. (2010). Understanding the complexity of cold tolerance in white clover

- using temperate gradient locations and a GWAS approach. *The Plant Genome*, 11, <https://doi.org/10.3835/plantgenome2017.11.0096>
- Jia, C., Zhao, F., Wang, X., Han, J., Zhao, H., Liu, G., & Wang, Z. (2018). Genomic prediction for 25 agronomic and quality traits in alfalfa (*Medicago sativa*). *Frontiers in Plant Science*, 9, 1220. doi:<https://doi.org/10.3389/fpls.2018.01220>
- Lamb, J. F., Sheaffer, C. C., Rhodes, L. H., Sulc, R. M., Undersander, D. J., & Brummer, E. C. (2006). Five decades of alfalfa cultivar improvement: impact on forage yield, persistence, and nutritive value. *Crop Science*, 46, 902-909. doi:<https://doi.org/10.2135/cropsci2005.08-0236>
- Langmead, B., & Salzberg, S. (2012). Fast gapped-read alignment with Bowtie 2. *Nature Methods*, 9, 357-359. doi:<https://doi.org/10.1038/nmeth.1923>
- Li, X., & Brummer, E. C. (2012). Applied genetics and genomics of alfalfa breeding. *Agronomy*, 2, 40-61. doi:<https://doi.org/10.3390/agronomy2010040>
- Li, X., Acharya, A., Farmer, A. D., Crow, J. A., Bharti, A. K., Kramer, R. S., . . . Brummer, E. C. (2012). Prevalence of single nucleotide polymorphism among 27 diverse alfalfa genotypes as assessed by transcriptome sequencing. *BMC Genomics*, 13, 568. doi:<https://doi.org/10.1186/1471-2164-13-568>
- Li, X., Wei, Y., Acharya, A., Hansen, J. L., Crawford, J. L., Viands, D. R., . . . Brummer, E. C. (2015). Genomic prediction of biomass yield in two selection cycles of a tetraploid alfalfa breeding population. *The Plant Genome*, 8. doi:<https://doi.org/10.3835/plantgenome2014.12.0090>
- Li, X., Wei, Y., Acharya, A., Jiang, Q., Kang, J., & Brummer, E. C. (n.d.). A saturated genetic linkage map of autotetraploid alfalfa (*Medicago sativa* L.) developed using genotyping-by-sequencing is highly syntenous with the *medicago truncatula* genome.

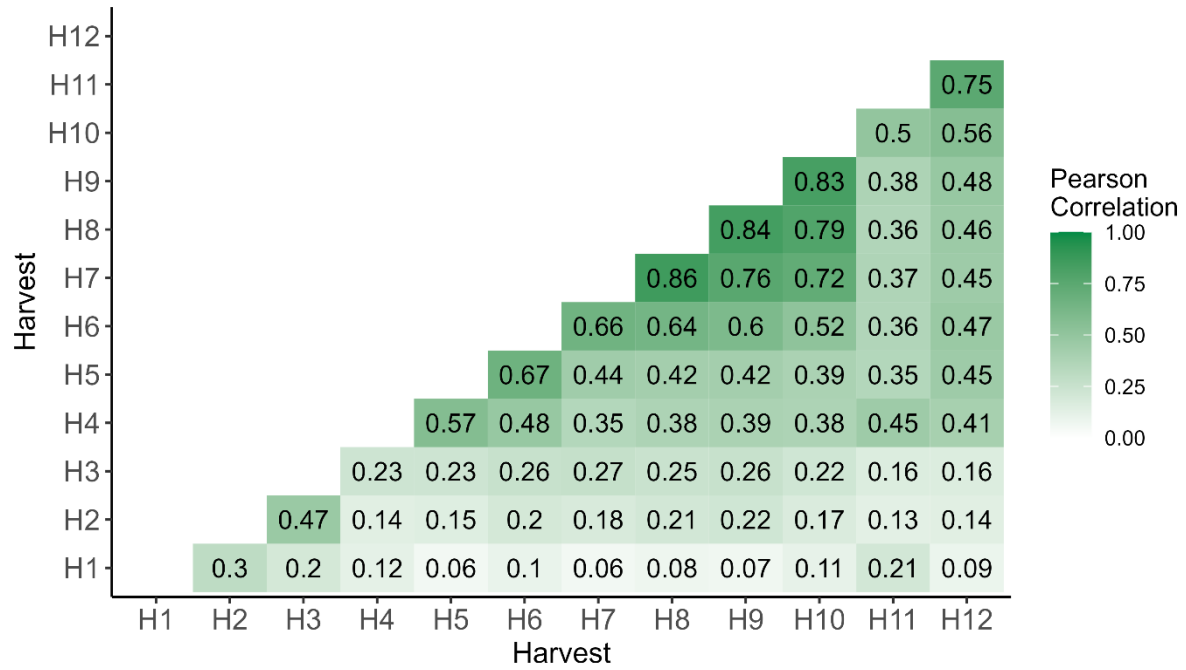
- Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet journal*, 17(1), 10-12. doi:<https://doi.org/10.14806/ej.17.1.200>.
- Meuwissen, T. H., Hayes, B. J., & Goddard, M. E. (2001). Prediction of total genetic value using genome-wide dense marker maps. *Genetics*, 157(4), 1819-1829. doi:<https://doi.org/10.1093/genetics/157.4.1819>
- National Oceanic and Atmospheric Administration (NOAA). (2023). Climate Monitoring. doi:<https://www.ncei.noaa.gov/access/monitoring/products/>
- Putnam, D. H., Summers, C. G., & Orloff, S. B. (2007). Alfalfa production systems in California. In C. G. Summers, & D. H. Putnam (Eds.), *Irrigated Alfalfa Management for Mediterranean and Desert Zones* (pp. 1-18). Oakland: University of California Agriculture and Natural Resources Publication 8287. doi:<http://alfalfa.ucdavis.edu/IrrigatedAlfalfa>
- R Development Core Team. (2018). R: a language and environment for statistical computing. R *Foundation for Stat. Comput.* Retrieved from <https://www.R-project.org/>
- RStudio Team. (2020). RStudio: Integrated Development for R. doi:<http://www.rstudio.com/>
- Shen, C., Du, H., Chen, Z., Lu, H., Zhu, F., Chen, H., . . . Wang, T. (2020). The chromosome-level genome sequence of the autotetraploid alfalfa and resequencing of core germplasms provide genomic resources for alfalfa research. *Molecular Plant*, 13, 1250-1261. doi:<https://doi.org/10.1016/j.molp.2020.07.003>
- United States Department of Agriculture (USDA). (2023). National Agricultural Statistics Service Quick Stats. doi:<https://quickstats.nass.usda.gov/>
- VanRaden, P. M. (2008). Efficient methods to compute genomic predictions. *Journal of Dairy Science*, 91(11), 4414-4423. doi:<https://doi.org/10.3168/jds.2007-0980>

Voss-Fels, K. P., Cooper, M., & Hayes, B. J. (2018). Accelerating crop genetic gains with genomic selection. *Theoretical and Applied Genetics*, *132*, 669-686. doi:<https://doi.org/10.1007/s00122-018-3270-8>

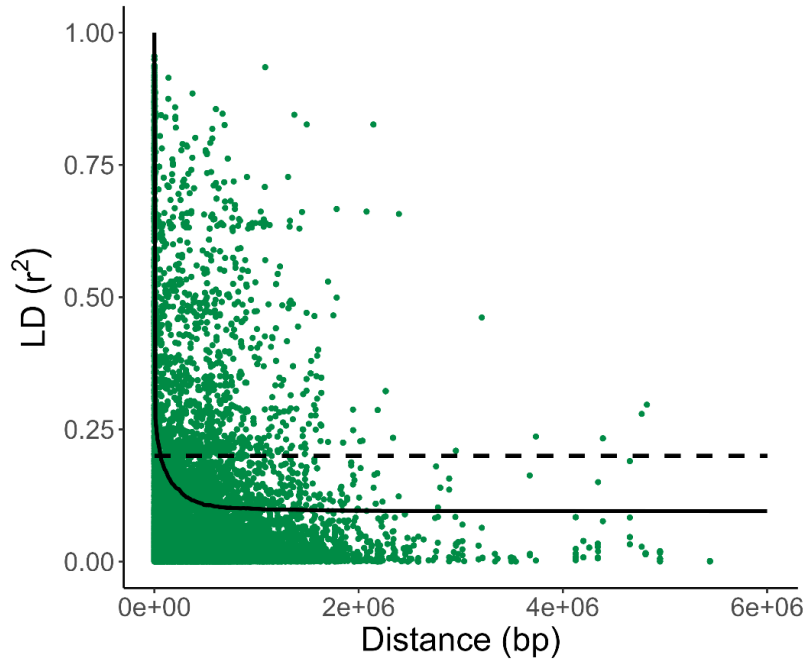




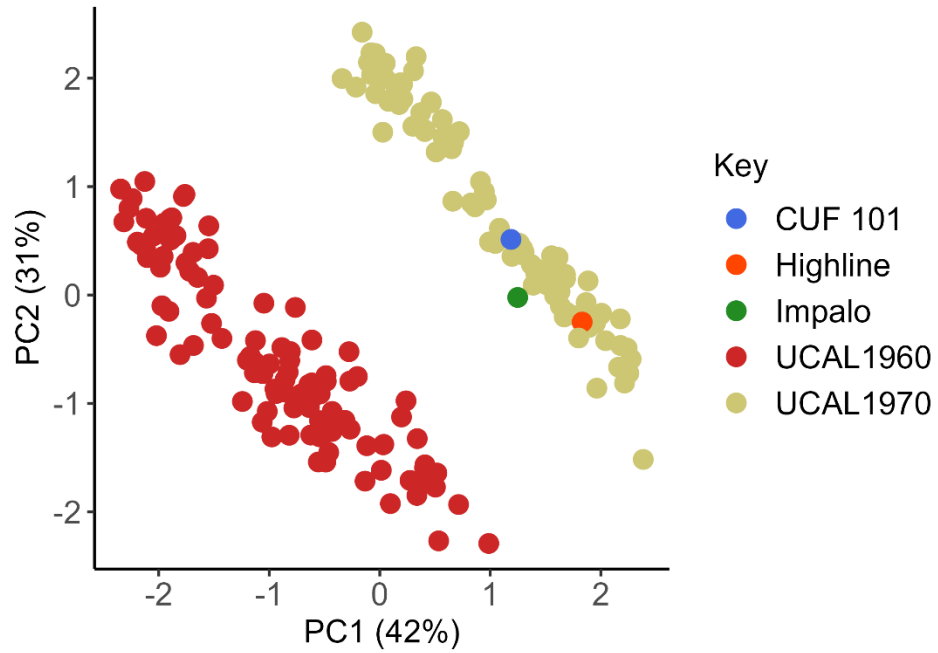
**FIGURE 1** Dry matter yield (kg DM ha<sup>-1</sup>) among alfalfa families across all 12 harvests.



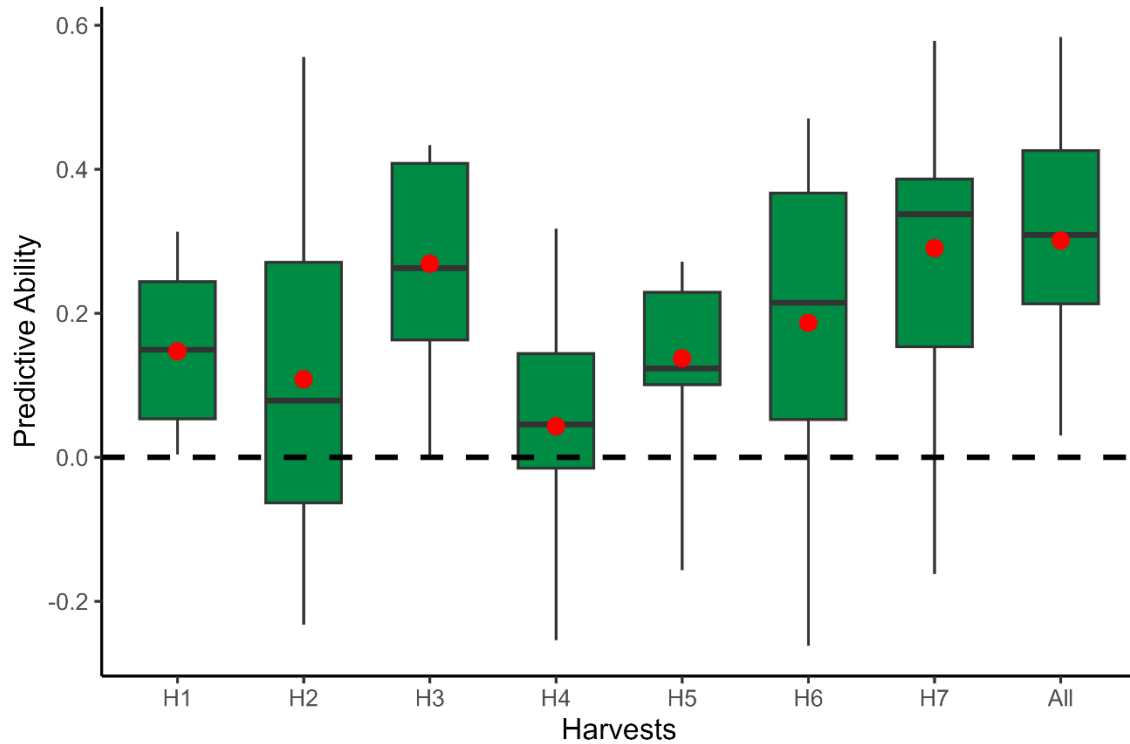
**FIGURE 2** Pearson correlation between phenotypic means for dry matter yield (DMY) across 12 harvests



**FIGURE 3** Linkage disequilibrium (LD) decay between SNP pairs. The LD decay over physical distance was determined as the mean distance at the LD threshold of  $r^2 = 0.2$ .



**FIGURE 4** Principal component analysis (PCA) of the genotype matrix showing the first two principal components that together explain 73% of the variation.



**FIGURE 5** Predictive ability for forage dry matter yield (DMY) for each individual harvest in the first full year of production and overall predictive ability from all seven harvests. Predictive ability was measured using Pearson correlation between the genomic estimated breeding value and phenotypic best linear unbiased prediction (BLUP) for all 193 alfalfa half-sib families. Red dots represent the average.

# Chapter 4

## Remote Sensing for Biomass Yield in Forage Breeding Plots

### 1 | INTRODUCTION

Yield is the most important trait for profitable forage production, yet the rate of genetic gain for dry matter yield (DMY) in perennial forage crops (0.25-0.7% per year) is lower than the main cereal crops (1.3% per year) and has been essentially zero in alfalfa over the past 30 years (Lamb et al., 2006; Ray et al., 2013; Brummer & Casler, 2014; Gebremedhin et al., 2019). Limited resources, low heritability, significant genotype by environment interaction ( $G \times E$ ) and long selection cycles limit the rate of genetic gain in perennial forages in comparison to many annual food and feed crops (Li & Brummer, 2012).

Improvement of perennial forages is typically carried out through recurrent phenotypic selection with or without progeny testing to accumulate desired alleles at high frequency in a population (Casler & Brummer, 2008). Ideally the number of families to be evaluated is very large, particularly with the advent of modern breeding methodology such as genomic selection. In reality, breeders must strike a balance between the available resources and the size and scope of breeding trials. Phenotypic evaluation of perennial forage traits requires significant investment of land, labor, and capital. Forage DMY and dormancy in alfalfa are two crucial traits that require significant resources to phenotype. The standard test for fall dormancy in alfalfa requires height measurements for each trial entry 25-30 days after the final harvest, often across multiple environments and years (Teuber et al., 1998). To accurately assess forage yield, experimental units must be harvested, dried,

and weighed to estimate dry matter content across multiple harvests and years, resulting in up to 40 total harvests over the lifetime of a trial (Biswas et al., 2021).

Further complexity is added to the breeding of perennial forages considering the diversity of evaluations trials often used, ranging from single plant evaluations to transplanted rows, seeded rows, or solid seeded swards. The choice depends on the traits of interest, the number of genotypes or families being evaluated, seed quantity, and the capital and labor resources available to the breeder, with most programs using a combination of sown and transplanted trials (Figure 1). Transplanted family rows are the most common as they are a cost-effective method of evaluating large numbers of trial entries for traits with high heritability. They are commonly used to screen populations for resistance or tolerance to various pests and diseases, investigating growth habit, dormancy, flowering time, and forage quality (Casler & Brummer, 2008). In the past, forage yield has often been selected indirectly based on evaluation of vigor on spaced plants or short family rows (Li & Brummer, 2012). Although a useful method for evaluating other important, highly heritable traits, a poor correlation exists between these assessment methods and yield in a commercial setting (Annicchiarico, 2006; Casler, 2008; Casler & Brummer, 2008).

Large sown plots are commonly used for variety trials. These trials require large quantities of seed, cover a large area, and provide phenotypic data for relatively few trial entries. In a breeding program, these trials are typically used to compare advanced breeding populations to released cultivars for key traits such as stand establishment, DMY, forage quality, flowering time, and dormancy. Although useful for obtaining phenotype data that well represents a commercial forage operation, it is usually not feasible to evaluate hundreds of families in this way.

Transplanted mini-sward plots provide a compromise between family rows and large sown plots. They seek to provide a better estimation of forage DMY than family rows without the need for large quantities of seed or significant land area that large sown plots require. In these trials a

small number of plants (20-50 plants per plot) are planted close to one another to mimic the competition observed in commercial forage stands.

In recent decades, remote sensing has been widely adopted in agricultural research (Maes & Steppe, 2019), offering a plethora of non-destructive vegetative data with massively reduced labor requirements. Remote sensing has the potential to address the lack of yield improvement in alfalfa and increase the rate of genetic gain for yield in other perennial forages by enabling breeders to greatly increase the size of trials without the associated increase in labor costs. This is particularly important for breeding programs looking to use genomic selection, where the size of the training population is a key component of predictive ability (Li & Brummer, 2012). Remote sensing techniques have been shown to enable accurate estimation of biomass yield in alfalfa at the field level (Feng et al., 2020), and at the large plot level in breeding trials (Cazenave et al., 2019; Tang et al., 2021). However, its accuracy has not been widely reported across the range of plot types used in forage breeding or for estimating fall dormancy in alfalfa.

The overall objective of this research project was to assess the accuracy of drone-based remote sensing versus traditional phenotyping for forage biomass yield and alfalfa fall dormancy across a variety of plot types used in perennial forage breeding. The goal is to give breeders the ability to evaluate a wider range of material without the associated increase in labor and costs. In addition, we aim to provide recommendations for researchers looking to incorporate similar technology into their breeding programs.



## 2 | MATERIALS AND METHODS

### 2.1 | Trial location

This experiment was carried out across several trials previously established as part of the UC Davis forage breeding program located on the UC Davis Plant Sciences Farm in Davis, CA (38°52' N, 121°77' W; 497 mm average annual rainfall) on a Yolo silt clay loam (fine-silty, mixed, superactive, thermic Fluventic Haploxerepts). It is a Mediterranean environment with hot, dry summers, cool winters and moderate annual rainfall which falls predominantly in the cooler months from November-March (National Oceanic and Atmospheric Administration [NOAA], 2023). Soil tests were conducted prior to planting to adjust P, K, and pH according to soil test recommendations. The trials consist of three alfalfa breeding trials and a forage grass variety trial.

### 2.2 | 2018 alfalfa dormancy trial – transplanted rows

The trial consisted of 72 released cultivars, experimental cultivars, germplasm populations, and eleven standard test check cultivars (Teuber et al., 1998). Plants were germinated in 128-cell flats in the greenhouse in February before transplanting to the field in April 2018. This experiment consisted of four replications laid out in a randomized complete block design. Plots consisted of a single row of 25 plants spaced 30 cm apart with a 90 cm gap between plots and 60 cm spacing between rows. Fertilizer was applied to maintain P and K at appropriate levels for a high yielding alfalfa stand, with weeds and insect pests monitored and control measures applied when necessary. Plants were initially watered using sprinkler irrigation until fully established, following which they were flood irrigated to satisfy full evapotranspiration (ET) requirements.

### 2.3 | 2018 alfalfa breeding trial – large sown plots

This trial contained 80 half-sib families of an experimental population UC2588 that had been selected for tolerance to lygus feeding. We had had sufficient seed of each family to plant solid seeded plots. This experiment was established following the NAAIC standard procedures for variety yield trials (Sheaffer et al., 1998). It consisted of two replications laid out in a randomized complete block design with ten rows and twenty ranges. Plots were 1 m x 3 m and were drilled using a small plot planter at a seeding rate of 15 kg ha<sup>-1</sup> with 1.5 m gaps between ranges. UC Impalo was sown as a border between ranges and around the exterior of the trial. As with the 2018 dormancy trial, crop nutrient demand, weeds and pests were monitored and adjusted when necessary. Sprinklers were used immediately after sowing to get the trial established, followed by flood irrigation to meet water demand.

### 2.4 | 2020 alfalfa genomic selection trial – transplanted mini-sward plots

This trial included a total of 198 entries of which 193 were half-sib families from two closely related elite UC Davis populations derived from various UC Davis germplasm that underwent selection for root rot and other stresses in El Centro and Davis, California. In addition, three cultivars: Highline, UC Impalo and CUF 101 were included as repeated checks and the remaining two entries were balanced bulks from each of the two populations (Chapter 3). The trial was sown in the greenhouse in March 2020 and transplanted two months later in early May at two locations on the UC Davis research farm in Davis, California. Each site has the same layout consisting of two replicates with 7 rows and 29 ranges for a total of 203 plots per rep, 812 plots overall. Plots consisted of 24 plants laid out in a regular 4 × 6 grid with 20 cm spaces between plants. There was a 30 cm space between rows and a 110 cm space between ranges to allow room for mechanical harvesting. This trial was managed as a high-yielding alfalfa stand, soil tests were conducted each year, with amendments made

accordingly. The trial was established using sprinkler irrigation, which was switched to flood irrigation after plants were well established. Irrigation water was added to roughly match crop ET. Weeds were managed by a combination of manual removal and herbicides, and insect pests were monitored and controlled with insecticide application as necessary, primarily for alfalfa weevil control in spring.

## 2.5 | 2020 grass variety trial – large sown plots

A grass variety trial containing 88 cultivars (32 tall fescue, 28 orchard grass, 14 timothy, 7 meadow fescue, 3 tall wheatgrass, 2 reed canary grass, and 2 meadow brome grass) was sown in October 2020. Plots are 1.5 m x 4.5 m and were drilled using a small plot planter. Table 1 outlines the seeding rates used for each species. The trial was separated by species with two blocks of tall fescue, two blocks of orchard grass, one block of timothy and reed canary grass, and the remaining species in the final block. Each block contained four rows of plots with 14 ranges. The blocks are separated by borders (two passes of the small plot planter) of either tall fescue, timothy, or orchard grass to allow irrigation pipes to be laid across the field without lying on top of the plots. This trial was irrigated by sprinklers on a weekly to biweekly basis as needed to approximate ET demand. N, P and K levels were monitored, and fertilizer applied when necessary. N was applied at 100 kg ha<sup>-1</sup> in spring and again after first harvest.

## 2.6 | Ground-based data collection

Plant height measurements for the alfalfa fall dormancy standard test were measured following the protocol outlined by Teuber et al. (1998). Twenty-five days after the final fall harvest, the natural plant height was measured on each of the 25 plants per plot. Natural plant height was deemed to be the distance from the soil surface to the top of the tallest stem as the plant stands in the field (i.e.,

without lifting the stems upright). The measurements were then averaged over the whole plot to generate a single data point for each plot.

Biomass yield data were collected using a small self-propelled plot harvester. Harvests occurred in alfalfa when the field had reached 10% bloom (approximately every 28 days through the growing season) with the first harvest usually occurring in late March/April and the final harvest in October. For alfalfa trials, subsamples were taken during each harvest, weighed wet, dried for at least 4d at 60C, and weighed dry to adjust moisture percentage. Several subsamples were taken from each replication as composite samples from all entries, rather than for every entry, and the average dry matter was used to adjust the wet weights.

In the grass trial, harvests occurred when the most plots of tall fescue and orchardgrass had reached the late boot stage, with the first harvest in April and subsequent harvests every 6-8 weeks for a total of four harvests per year. All species were harvested at the same time for logistical reasons, even though this was likely not ideal for individual species (or indeed, specific cultivars of all species). All forage was clipped uniformly at 7.5 cm, weighted, and removed from the trial area. Subsamples were taken from every plot in the grass trials, weighed wet, dried for at least 4d at 60C, and weighed dry to adjust moisture percentage.

## 2.7 | Remote-sensing data collection

Prior to remote sensing data collection, the borders surrounding the trial and between plots were mown. Drone flights and preliminary image processing were conducted following methods modified from Parker et al. (2020). A Micasense RedEdge-M multispectral camera was mounted to a DJI Matrice 100 drone to collect field imagery. Flights occurred at solar noon the day prior to destructive harvests, and images were captured automatically using DJI Ground Station Pro. The drone was flown at an altitude of 15 m with 80 percent overlap between images. Raw images from each flight

were built into field-scale orthomosaics (RGB and normalized difference vegetation index [NDVI]) and digital surface models (DSMs) using Pix4Dmapper version 4.3.31.

The processed orthomosaics were loaded into QGIS version 3.22.1 for extraction of data at the plot level. First a grid layer was generated using the create grid function of QGIS overlaying each plot (Figure 2, top right). Next a threshold layer was generated from the NDVI orthomosaic (Figure 2, bottom left) to differentiate between plants and soil. This threshold layer was then used to partition the DSM (Figure 2, bottom right) into two separate layers, a canopy DSM and a soil DSM that contained elevation information for each pixel. The zonal statistics plugin was then used to calculate the percent canopy area, mean canopy elevation, and mean soil elevation for each plot. From these statistics we calculated plant height by subtracting the average soil elevation from the average canopy elevation and a canopy volume index by multiplying the average plant height by the percent canopy area.

## 2.8 | Statistical analysis

All analyses were performed using R statistical software (v4.1.0; R Core Team 2021). A simple linear model was used to analyze alfalfa plant height from both ground measurements and remote sensing estimates in the 2018 dormancy trial. Least squares means for each entry in the trial were calculated using the emmeans package by Lenth (2021). The Pearson correlation coefficient was then used to compare the two forms of data collection.

In the remaining trials, the Pearson correlation coefficient was again used to compare ground measured fresh weight with remote sensing estimates for biomass volume.

### 3 | RESULTS

#### 3.1 | Biomass volume

We have flight data from a single harvest in the grass trial and alfalfa dormancy trial, from six harvests in the large sown alfalfa trial and from eleven harvests in the transplanted mini-sward alfalfa trial. The accuracy of drone-based remote sensing varied across the range of plot types and forage species, but overall, a high correlation between the two forms of data collection was observed (Figure 3). The Pearson correlation between the drone estimated volume index and ground-measured plot fresh weight was greatest in the transplanted family rows used in the 2018 alfalfa fall dormancy trial (0.92) and was the lowest in the transplanted mini-sward plots used in the 2020 genomic selection trial (0.74). In the large sown plots, there was a greater correlation in the grass trial (0.85) than the alfalfa trial (0.80), although remote sensing was highly correlated with the ground measurements in both.

The relationship between remote sensing and ground data appears to be linear in all instances except for the transplanted mini-sward plots. In this trial, differences in plots with high fresh weight were not as well identified by the drone as they were in plots with low fresh weight or in the other types of plots. In addition, there appears to be several instances of high biomass plots registering low volumes from the drone imagery, likely due to lodging.

#### 3.1 | Plant height

The Pearson correlation between least squares means for drone estimated plant height and measured plant height (Figure 4) is extremely high at 0.95. The check cultivars (highlighted in red in figure 4) generally performed as expected with the exception of the most extreme non-dormant check. The check appears as an outlier in Figure 4 (circled in red). Its reported fall dormancy rating is 11;

however, it aligns between the cultivars with reported ratings of 6 and 7. Additionally, the same check registered a much lower height index from the drone imagery in relation to its measured height.

#### 4 | DISCUSSION

The process of phenotyping in plant breeding is expensive and laborious. In perennial forage breeding programs, which often have limited resources, evaluating large breeding populations is challenging due to repeated harvests across multiple years. Drone-based remote sensing offers a fast and effective method of assessing a large amount of material with little increase in labor.

Incorporation of such technology in a breeding program enables breeders to increase selection intensity by increasing the scale of breeding trials and thus improving the rate of genetic gain or to replace manual measurements for laborious phenotyping tasks. Drone estimated biomass volume serves as an effective proxy for biomass yield across a range of perennial forage breeding plot types as demonstrated in this study. Optimizing trials for the collection of remote sensing data and improving the high throughput phenotyping data analysis and curation pipelines could make the incorporation of this technology into breeding programs routine and could help to address the low rate of genetic gain observed in most perennial forage crops.

Fall dormancy is a crucial trait in alfalfa that provides growers with information related to the potential of a cultivar to perform in specific environments. Alfalfa breeders must be aware of the degree of fall dormancy in their experimental populations to ensure their cultivars fit the target environments. The standard test for fall dormancy in alfalfa (Teuber et al., 1998) has a significant labor requirement and necessitates growing a dedicated trial that only provides dormancy data. Remote sensing estimates of plant height align very closely with ground-based plant height

measurements for a single location and season and may be a more accurate way to assess plant height due to the large number of data points for each plant. When creating an orthomosaic from drone images, the digital surface model includes height information for each pixel; with the camera used in this trial and at a height of 15 m, each plant was represented by hundreds of data points per plant rather than a single measurement by hand of the tallest point of the plant. This will result in a more representative reading for the height of each plant and subsequently a better estimation of fall dormancy. We have previously shown that alfalfa fall dormancy characterization in sward plots is equivalent to the spaced plants used in the standard test (Newell et al., 2023). Our results here suggest that modifying the fall dormancy standard test to enable remote-sensing height data collected as a matter of course in variety trials could be feasible, saving plant breeders time and money in evaluating dormancy response and providing growers with a more precise dormancy estimate.

Although remote sensing has significant potential in forage breeding programs as they currently exist, a number of design and management modifications could be made to future breeding trials to maximize the quality of data being collected. First, anything that affects the soil level or plant height will be reflected in the data. Lodging was the most significant problem that we encountered over the course of this study and is a major issue concerning plant height or volume estimates from multispectral aerial imagery as it results in the underestimation of true biomass. Selecting a harvest interval that minimizes lodging is one method to mitigate this issue. Additionally, breeders can adjust flight schedules in anticipation of weather events that may result in lodging, such as high winds or heavy rain. As well as causing lodging, wind can influence the quality of aerial images. Plants that are moving in the wind may cause anomalies when stitching the raw images into an orthomosaic; therefore, weather monitoring is an important consideration in remote sensing as well as having a flexible flight schedule. Machinery traffic and mammalian pests are the main cause



of soil level issues that we encountered. Wheel ruts from machinery may lead to overestimation of height and biomass, while mounding from mammalian pests such as gophers and ground squirrels will have the opposite effect. Thus, controlling mammalian pests and avoiding traffic on trials when the ground is soft will result in better remote sensing estimates.

The method of data collection used in this study requires some reference to ground level within each plot and designing trials to enable clear ground level identification will improve the data collection process. Large gaps between plots will ensure that a ground reference can be found throughout the trial. A system that we recommend is that used in our grass trial, where alleys were mown between plots prior to flying. This has the advantage of maintaining the selection pressure from competition that plants would experience in a commercial field, while also providing a solid baseline for ground level. It also has the additional benefits of limiting the impact of traffic on the soil level and controlling weeds surrounding the plots. Finally, although not essential, ensuring trials are arrayed on a regular grid with straight lines and even plot sizes will streamline the data extraction process. Overlaying a grid in QGIS requires significant manual adjustment for trial layouts that are not uniform so additional care when planning and planting a trial, or ideally, using a GPS equipped planter will greatly simplify the pipeline.

Though beyond the scope of this paper, a host of alternative applications for remote sensing data beyond plant height and volume estimation can be imagined. The non-destructive nature of remote sensing means that a breeder could measure the entire growth cycle of forage crops to make better informed selection decisions. There are wide range of vegetation indices other than NDVI to highlight various properties of vegetation that are not observable to the naked eye. Forage quality is another key trait that breeders must evaluate for which different vegetation indices may prove useful. Remote sensing imagery also serves as a digital archive of trials that can be revisited to better understand trends in trial development over time.

Despite the substantial range of applications for remote sensing data in perennial forage breeding, there are a number of limitations. High throughput data collection, storage, and processing require hardware and software investment and the knowledge to develop an analytical pipeline to extract actionable information. Also, weather plays a substantial role in data collection (as it does in traditional phenotyping methods). Finally depending on the camera and drone, there may be a large initial investment to get set up.

Remote sensing offers a promising method to reduce the costs of phenotyping in perennial forage breeding programs with the accurate estimation of important traits. The results from this study suggest that breeders could increase the size of breeding trials without a proportional increase in labor and consequently increase the rate of genetic gain for forage yield. Breeders also have a new method of assessing fall dormancy in alfalfa that requires significantly less labor than traditional phenotyping. Ground-measurements supplemented with remote sensing data opens the door for smaller, resource limited breeding programs to adopt new methodology such as genomic selection to help bridge the gap in genetic gain between perennial forages and alternative crops thus ensuring continued inclusion in crop rotations.

## 5 | ACKNOWLEDGEMENTS

We thank the US Alfalfa Farmer Research Initiative and the California Crop Improvement Association for support of this research to ECB and PGG Wrightson Seeds for a fellowship supporting MF.

- Annicchiarico, P. (2006). Prediction of indirect selection for seed and forage yield of lucerne based on evaluation under spaced planting. *Plant Breeding*, *125*, 641-643.  
doi:<https://doi.org/10.1111/j.1439-0523.2006.01295.x>
- Biswas, A., Andrade, M. H., Acharya, J. P., de Souza, C. L., Lopez, Y., de Assis, G., . . . Rios, E. F. (2021). Phenomics-assisted selection for herbage accumulation in alfalfa (*Medicago sativa* L.). *Frontiers in Plant Science*, *12*. doi:<https://doi.org/10.3389/fpls.2021.756768>
- Brummer, E. C., & Casler, M. D. (2014). Cool-season forages. In S. Smith, B. Diers, J. Specht, & B. Carver (Eds.), *Yield Gains in Major U.S. Field Crops* (pp. 33-52). Madison, WI: ASA, CSSA, SSSA.
- Casler, M. D. (2008). Among-and-within-family selection in eight forage grass populations. *Crop Science*, *48*, 434-442. doi:[10.2135/cropsci1990.0011183X003000010004x](https://doi.org/10.2135/cropsci1990.0011183X003000010004x)
- Casler, M. D., & Brummer, E. C. (2008). Theoretical expected genetic gains for among-and-within-family selection methods in perennial forage crops. *Crop science*, *48*(3), 890-902.  
doi:<https://doi.org/10.2135/cropsci2007.09.0499>
- Cazenave, A. B., Shah, K., Trammell, T., Komp, M., Hoffman, J., Motes, C. M., & Monteros, M. J. (2019). High-throughput approaches for phenotyping alfalfa germplasm under abiotic stress in the field. *The Plant Phenome Journal*, *2*, 1-13. doi:<https://doi.org/10.2135/tppj2019.03.0005>
- Feng, L., Zhang, Z., Ma, Y., Du, Q., Williams, P., Drewry, J., & Luck, B. (2020). Alfalfa yield prediction using UAV-based hyperspectral imagery and ensemble learning. *Remote Sensing*, *12*(12), 2028. doi:<https://doi.org/10.3390/rs12122028>

- Gebremedhin, A., Badenhorst, P. E., Wang, J., Spangenberg, G. C., & Smith, K. F. (2019). Prospects for measurement of dry matter yield in forage breeding programs using sensor technologies. *Agronomy*, 9(2), 65. doi:<https://doi.org/10.3390/agronomy9020065>
- Lamb, J. F., Sheaffer, C. C., Rhodes, L. H., Sulc, R. M., Undersander, D. J., & Brummer, E. C. (2006). Five decades of alfalfa cultivar improvement: impact on forage yield, persistence, and nutritive value. *Crop Breeding, Genetics and Cytology*, 46, 902-909. doi:<https://doi.org/10.2135/cropsci2005.08-0236>
- Lenth, R. V. (2021). emmeans: Estimated Marginal Means, aka Least-Squares Means. doi:<https://CRAN.R-project.org/package=emmeans>
- Li, X., & Brummer, E. C. (2012). Applied genetics and genomics of alfalfa breeding. *Agronomy*, 2, 40-61. doi:<https://doi.org/10.3390/agronomy2010040>
- Maes, W. H., & Steppe, K. (2019). Perspectives for remote sensing with unmanned aerial vehicles in precision agriculture. *Trends in Plant Science*, 24, 152-164. doi:[10.1016/j.tplants.2018.11.007](https://doi.org/10.1016/j.tplants.2018.11.007)
- National Oceanic and Atmospheric Administration (NOAA). (2023). Climate Monitoring. doi:<https://www.ncei.noaa.gov/access/monitoring/products/>
- Parker, T. A., Palkovic, A., & Gepts, P. (2020). Determining the genetic control of common bean early-growth rate using unmanned aerial vehicles. *Remote Sensing*, 12(11), 1748. doi:<https://doi.org/10.3390/rs12111748>
- Ray, D. K., Mueller, N. D., West, P. C., & Foley, J. A. (2013). Yield trends are insufficient to double global crop production by 2050. *Plos One*, 8(6). doi:<https://doi.org/10.1371/journal.pone.0066428>
- RStudio Team. (2020). RStudio: Integrated Development for R. doi:<http://www.rstudio.com/>

- Sheaffer, C., Undersander, D., Bowley, S., Johnson, D., Martin, N., & McCaslin, M. (1998). Forage yield testing guidelines. In *Standard Tests to Characterize Alfalfa Cultivars*. North American Alfalfa Improvement Conference (NAAIC).
- Tang, Z., Parajuli, A., Chen, C. J., Hu, Y., Revolinski, S., Medina, C. A., . . . Yu, L. (2021). Validation of UAV-based alfalfa biomass predictability using photogrammetry with fully automatic plot segmentation. *Scientific Reports*, *11*, 3336. doi:<https://doi.org/10.1038/s41598-021-82797-x>
- Teuber, L. R., Taggard, K. L., Gibbs, L. K., McCaslin, M. H., Peterson, M. A., & Barnes, D. K. (1998). Fall dormancy. In *Standard Tests to Characterize Alfalfa Cultivars*. North American Alfalfa Improvement Conference (NAAIC).
- United States Department of Agriculture (USDA). (2023). National Agricultural Statistics Service Quick Stats. doi:<https://quickstats.nass.usda.gov/>

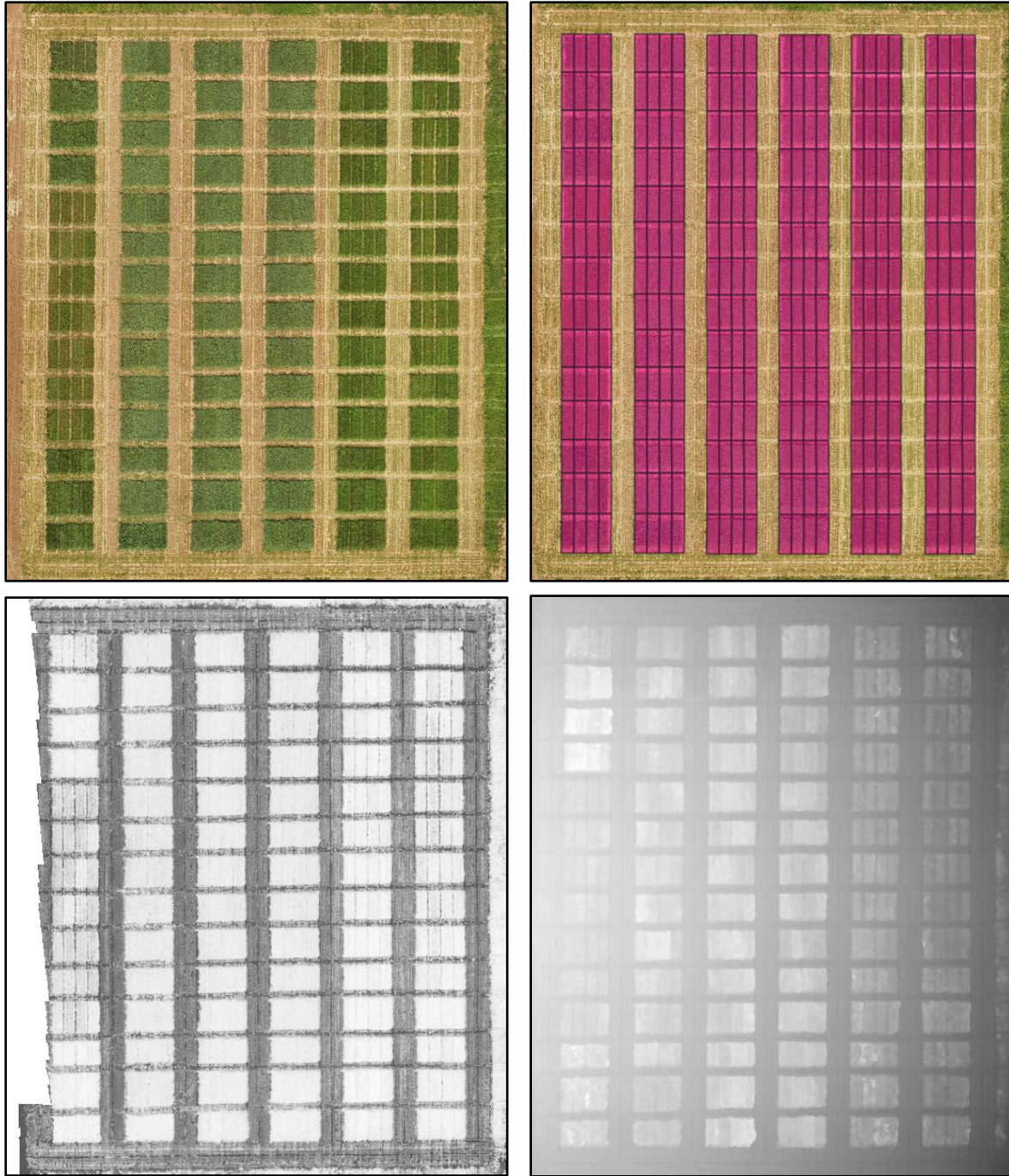


**FIGURE 1** Different types of alfalfa plots used in the UC Davis alfalfa breeding program: transplanted row plots (top left), large sown sward plots (top right), and transplanted mini sward plots.

**TABLE 1** Sowing rates for 2020 grass variety trial

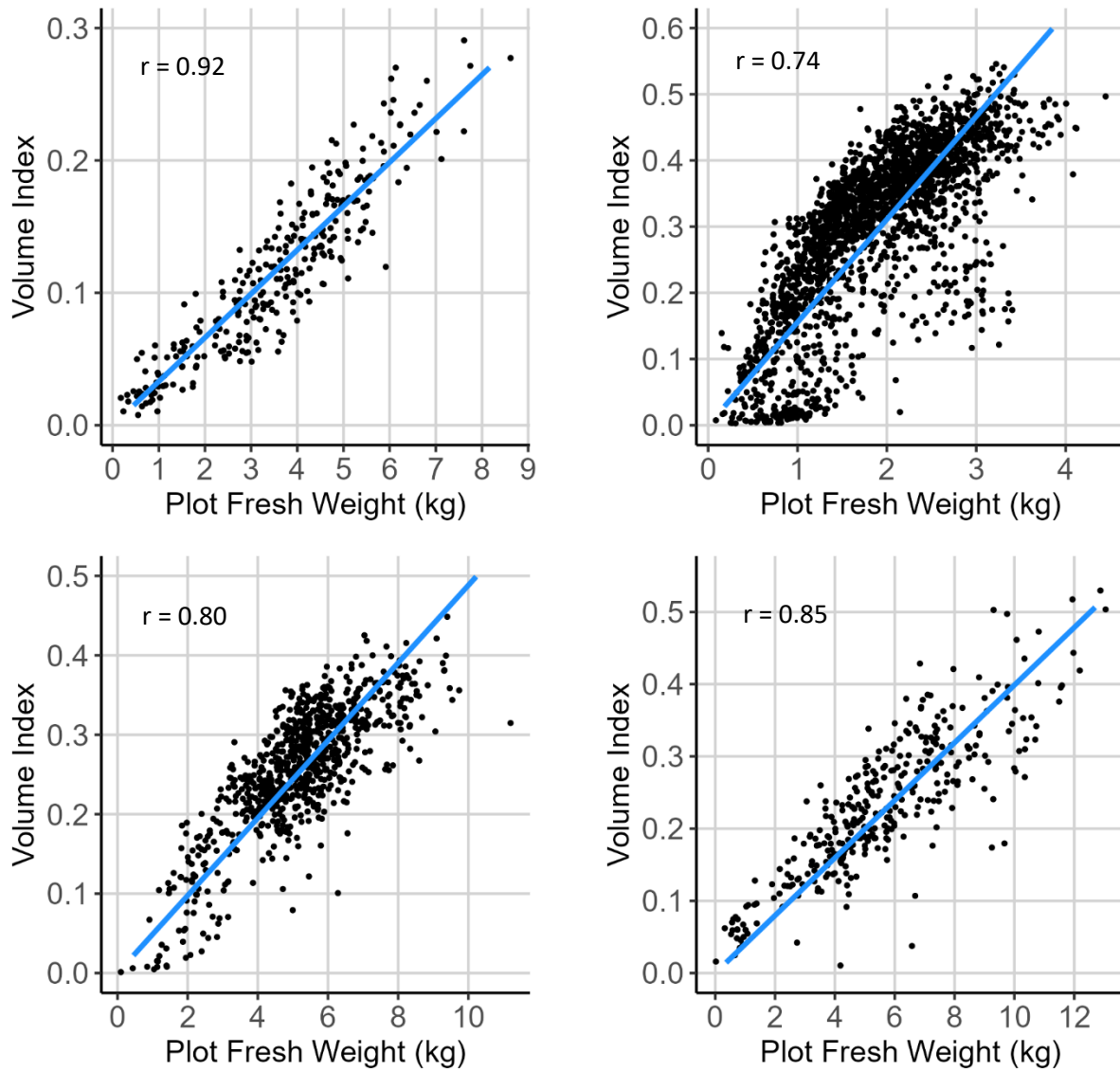
<b>Species</b>	<b>Seeding rate (kg PLS ha<sup>-1</sup>)</b>
Tall fescue ( <i>Festuca arundinacea</i> Schreb.)	22.4
Orchard grass ( <i>Dactylis glomerata</i> L.)	16.8
Timothy ( <i>Phleum pratense</i> L.)	5.6
Meadow fescue ( <i>Festuca pratensis</i> Huds.)	22.4
Tall wheatgrass ( <i>Thinopyrum ponticum</i> [Podp.] Z.-W. Liu & R.-C. Wang)	11.2
Reed canary grass ( <i>Phalaris arundinacea</i> L.)	5.6
Meadow brome grass ( <i>Bromus riparius</i> Rehm.)	9.0



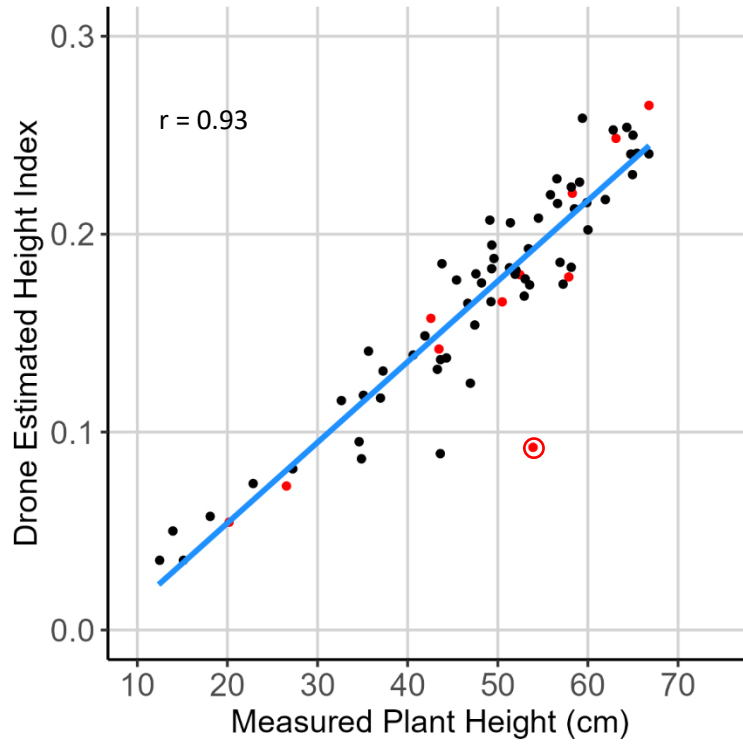


**FIGURE 2** Workflow for extracting biomass estimates from aerial imagery. True color image (top left), grid overlay to calculate statistics at the plot level (top right), Normalized difference vegetation index (NDVI) for differentiating between live biomass and ground level (bottom left), and digital surface model (DSM) containing relative pixel height to determine plot volume (bottom right).





**FIGURE 3** Measured fresh weight versus drone estimated volume for: alfalfa transplanted row plots (top left), alfalfa transplanted mini sward plots (top right), alfalfa large sown sward plots (bottom left), and grass large sown sward plots (bottom right)



**FIGURE 4** Least squares means of ground-measured plant height versus drone estimated height for alfalfa transplanted row plots. Red dots indicate the check cultivars/populations used in the standard test for alfalfa fall dormancy.